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PRINCIPAL INVESTIGATOR: Johnnye L. Lewis, Ph.D.
Graham Bench, Ph.D.
Fletcher F. Hahn, Ph.D.

CONTRACTING ORGANIZATION: University of New Mexico Health Science Center
Albuquerque, New Mexico 87131-5041

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Johnnye L. Lewis, Ph.D.
Graham Bench, Ph.D.
Fletcher F. Hahn, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of New Mexico Health Science Center
Albuquerque, New Mexico 87131-5041

E-Mail: jlewis@cybermesa.com

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13. ABSTRACT (Maximum 200 Words)

This study investigates the potential for inhaled uranium oxide (UO) aerosols to penetrate the nose-brain barrier, directly enter the central nervous system (CNS), distribute within the CNS, and result in slowly developing neurotoxicity. Substantial inhalation exposures to depleted uranium (DU) may have occurred during the GW in several scenarios of varying duration and airborne uranium concentration. Penetration of the nose-brain barrier can result in CNS deposition of metals even if the blood-brain barrier limits penetration of systemically circulating material. Nasal inflammation is examined as a modifying factor that could result in increased sensitivity to uranium uptake via penetration of the nose-brain barrier. Nephrotoxic and pulmonary effects are evaluated to determine whether CNS effects can occur at lower thresholds than nephrotoxic effects. Soluble and insoluble uranium as well as mixtures are being studied. Analysis of tissues following acute (15 min), high dose (500 mg/m^3) uranium inhalation shows that soluble uranium (UO_3) at those concentrations produces lethal kidney tubular necrosis in female animals, and that kidney toxicity resulting in uremic pneumonia in male and surviving female animals exposed to either DU oxides or soluble UO_3 results in a lung fibrosis that persists for at least one year post-exposure. In a small subset of animals, uranium can be detected in the olfactory bulbs of the brain following inhalation of a 1 mg/m^3 UO_2+UO_3 mixture for as little as 6 hours.

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Inhalation of Uranium Oxide Aerosols: CNS Deposition, Neurotoxicity and Role in Gulf War Illness -

Annual Report: September 2004

PI – Johnnye L. Lewis, Ph.D., DABT

Co-PIs

Graham Bench, Ph.D.

Fletcher Hahn, Ph.D.

INTRODUCTION

Purpose: The purpose of the overall project is to test the hypothesis that inhaled uranium–containing aerosols will enter the central nervous system (CNS) via olfactory transport, follow neuronal pathways to distal regions of the CNS, and ultimately result in neurodegeneration. The studies in Year 3 address metal uptake and neuronal damage after moderate or long term, moderate concentration exposures to uranium oxides. These studies examine the effects of inhaled uranium oxides both in a healthy rat model and one in which inflammation has been induced in the upper respiratory tract. Target organs to be examined include nasal and lung tissue, brain, spinal cord and kidney. A 6 months to one-year time course of response following the exposures will be determined with sacrifices scheduled on 0, 14, 30 and 180 days post-exposure or 0, 30, 180, and 360 days post-exposure, respectively. Results of these exposures will address all of the original hypotheses:

Hypothesis I. Inhalation of uranium aerosols during the Gulf War from combustion of DU containing weapons resulted in CNS deposition and subsequent neurodegeneration in a subset of those exposed.

Hypothesis II: Transient conditions such as inflammation compromised the olfactory epithelium and enhanced the entry of uranium and the subsequent development of neurodegeneration

Hypothesis III: Markers of neurodegeneration are correlated with the concentration and deposition of U within the CNS following inhalation exposure.

Hypothesis IV: The degree of and time-course of neurodegeneration are dose and exposure duration dependent.

These hypotheses will be tested for both short- and long-term exposure scenarios as differences related to exposure rate and dose may be critical to uptake, clearance, and ultimate neurotoxicity. We originally proposed the following four inhalation ***exposure scenarios***:

- ***Tank-Impact Scenario:***
  Acute (15 min) – high-level concentrations (500 mg/m^3)
- ***March-Through Scenario:***
  Short duration – moderate concentration (1 mg/m^3 - 6 hrs)
- ***Clean-Up Scenario:***
  Longer duration – moderate concentration (1 mg/m^3/ 6 hrs/ 30 days)
- ***Maintenance Scenario:***
  Long-term – low concentration (0.01 mg/m^3/ 6 hrs/ 30 days)

Results from Year 1-2 suggested that the Maintenance scenario above would not add further information to the tests of the hypotheses and that scenario has been dropped in favor of more in-depth analyses of the Clean-Up Scenario. As part of the approved re-scope of work (see below), the Maintenance scenario was replaced with a

- ***Re-exposure Scenario:***
  Long duration moderate concentration (1mg/m^3/ 6 hrs/ 30 days) 11 months after initial long or short duration – moderate concentration

BODY

Approved Scope of Work for Year 3

Year 1-2 Scope as a prelude to Year 3 tasks

During Year 1, rats will be exposed via nose-only inhalation for 15 minutes to aerosols of 1) insoluble UO_2; 2) soluble UO_3; 3) a mixture containing by weight 50% UO_2 and 50% UO_3; 4) TaO_2 (a negative control) at concentrations of 500 mg/m^3. A fifth group will be co-exposed to endotoxin (to induce inflammation) and the UO_2 + UO_3 mixture for 15 minutes. Thirty animals will be exposed in each group. Separate air-only and endotoxin-air exposed control groups will be employed as vehicle controls. For each group rats will be sacrificed in sets of 6 per group immediately following exposure, and at 30, 180 and 360 days post-exposure. Tissue analyses from the short-term exposures will begin in Year 1, but substantially carry over to Year 2.

Longer duration exposures will be performed in year 2 and 3. Serial sacrifices of rats exposed via nose-only inhalation for *8 hours* to aerosols of a mixture containing by weight 50% UO_2 and 50% UO_3 at a dose of 1 mg/m^3 will be performed. A separate air-only exposed control group will be employed as vehicle control. For each group rats will be sacrificed in sets of 6 per group immediately following exposure, and at 30, 180 and 360 days post-exposure. Serial sacrifices of rats exposed via nose-only inhalation for *30 days* to aerosols of a mixture containing by weight 50% UO_2 and 50% UO_3 at a dose of 1 mg/m^3 will be performed. A separate air-only exposed control group will be employed as vehicle control. For each group rats will be sacrificed in sets of 6 per group immediately following exposure, and at 30, 180 and 360 days post-exposure. Tissue analyses for longer duration exposures will begin in Year 2, but substantially carry over to Year 3

Year 3 Scope

Quantitation of metals in nose and brain tissues will be performed with Atomic Absorption Spectrophotometry (AAS) and microbeam Proton Induced X-ray Emission (μ-PIXE). Immunohistochemistry (IHC) of heat shock proteins (HSP) will be done on nasal and representative brain tissues. Brain tissues will also be examined using IHC for the persistence of tyrosine hydroxylase - containing neurons and for the persistence of NeuN labelling. Neuroinflammation will be monitored by GFAP (glial fibrillary acidic protein) -IHC. A marker of neuronal degeneration (Fluorojade histochemistry) and a preliminary indicator of the neuronal apoptosis (TUNEL) will also be examined in sections from brains showing U deposition. If data from any of these categories indicates that there is neurodegeneration, specific brain areas such as the substantia nigra (SN) and anatomically-linked areas will be studied in greater detail using IHC directed against other DA cell markers such as the postsynaptic receptors D1 and D2 within the substantia nigra, caudate putamen and olfactory bulbs. Immunoinflammatory markers (IL-1, IL-6, TNFa and their receptors) and immunohistochemistry to detect 4-hydroxynonelation of nigral dopaminergic neurons will also be performed.

One kidney and one lung from each sacrificed animal will be analyzed by AAS for inhaled metal content. The other lung and kidney from each sacrificed animal will undergo histopathological examination. Data from the kidney analyses will allow us a measure of potential nephrotoxicity and will afford a comparison of inhalation data with nephrotoxic

effects arising from the study of DU implants. Data from lung will afford an assessment of any pulmonary damage resulting from the inhalation exposures.

Multivariate analyses will be used to examine differences between the groups in both the concentrations of metal localized within different brain regions, and the levels of the indicators of neurodegeneration and other markers analyzed. In conditions where the N is not sufficient for this parametric analysis, nonparametric and qualitative analyses will be utilized instead.

A re-scope of work was approved in 2004

We propose replacing the Maintenance Scenario at this time with additional animals in the "Clean-up Scenario" exposures to clarify and expand positive findings seen to date. Tissue from these animals will be used chiefly to increase our understanding of mechanisms underlying toxicity by looking at early signs of DNA damage and repair, as well as markers of oxidative stress likely to accompany uranium entry into the CNS. These studies will be done in collaboration with Fernando Cardozo-Pelaez, Ph.D., at the University of Montana who has developed methodologies and published in this area on metal neurotoxicity.

In addition, we propose examining the spinal cords in the exposed animals for markers of damage associated with amyotrophic lateral sclerosis, a neurodegenerative disease recently noted to occur at increased prevalence in Gulf War veterans.

We propose to re-expose animals previously exposed and currently being held for long-term survival. Because uptake was limited in the early time-points, reexposing these animals and comparing uptake and markers of damage between those with single and repeated exposures will provide excellent pilot data on potential increased risk of multiple exposure and indicate whether this avenue should receive further attention.

Finally, we propose to look at U uptake in kidneys to explore the gender difference seen with the high-dose exposures. Females were more sensitive to lethal effects at the high dose exposures, and to date more females have died in the lower-dose exposures as well. Because of the observation of increased sensitivity in the females, we would like to determine, using more sensitive analytical methods for Uranium in organ tissues (ICP-MS), whether we see differential uptake in females that could underlie the increased sensitivity to kidney toxicity.

Progress on Year Three Scope

The following section is organized first by exposure scenario and then by task identified in the original scope of work documented above. The relevant section of the scope, quoted and in bold italics, begins each description of work.

A. "Tank impact scenario" – acute, high dose exposure

Exposures and sacrifice of 0, 30, 180 and 360 day survival times was completed in Years 1-2. Results from analysis of brain uranium uptake and brain immunohistochemistry as well as the initial analysis of kidney and lung toxicity have been reported previously.

TASK 1: ***One kidney and one lung from each sacrificed animal will be analyzed by AAS for inhaled metal content.***

Note: Kidneys from animals sacrificed 30 days after acute exposure to UO_2, UO_3, UO_2 + UO_3 or DUOx were analyzed in Year 2. The uranium levels were below the detectable level for all samples. In order to increase probability of detectable uranium levels in our samples, kidney tissue from UO_3 exposed female animals that died between the 0 and 30 day sacrifice times was pooled in sets of 2-5 animals (see Table1). In addition, as noted in last year's progress report, the method was changed from AAS to ICP-AES. These animals showed significant lesions in the kidneys to be the cause of death, and therefore, the concentration of uranium in the kidneys becomes an important factor in understanding toxic responses in the kidney.

Methods:
Kidney from each animal was prepared for analysis by dry ashing at 550°C. To ensure confidence in the analytical accuracy of the data, tissues were ashed for 48 hours. The ashed samples were digested for time periods of 1-2 days in 6 ml of nitric acid and metal content was analyzed using ICP-AES. Analysis of standard samples with known uranium content produced a detection limit of 0.3 µg/g dry weight (or 0.3 mg/kg dry weight).

Results:

The uranium concentrations in kidneys from early death UO_3-exposed rats were substantially higher than in the scheduled sacrifice animals analyzed in Year 2. Table 1 illustrates U content per mg dry kidney weight. These data are based on pooled tissues as noted above.

Table 1. Uranium content in female UO_3-exposed early death rats.

Days after exposure	Number of animals	Uranium concentration (mg /kg dry weight) ± SD
6	5	34.2 ± 2.1
7	3	34.6 ± 1.7
8	3	24.6 ± 1.7
10 and 13	2	23.4 ± 1.3

The results are consistent with the kidney pathology previously reported as the cause of death in these animals. The decreasing concentrations observed from the day 6 to the day 10-13 animals are also consistent with the inability to detect uranium in the kidneys of animals surviving to the 30-day sacrifice time reported in last year's progress report. In summary, the data indicate an early kidney deposition of uranium increasing from an average of 10 mg/kg at 4 hr post-exposure, probably peaking at 6-7 d post exposure (time of death) at lethal concentrations (particularly to females) greater than 30 mg/kg, followed by clearance from the kidneys in those animals surviving the initial insult. By the originally scheduled 30 day sacrifice, kidney concentrations had been reduced to non-detect levels observed in the previously reported analysis.

TASK 2: ***One kidney and one lung from each sacrificed animal will undergo pathological examination***

The analysis of kidney and lung pathology from all rats in the acute exposure is now complete. A detailed report is attached as Appendix 1 (manuscript in preparation) and a summary of methods and results is found below.

Methods:

A necropsy was performed on all rats dying, sacrificed in a moribund condition or sacrificed at scheduled times. Organs of the cranial, thoracic, and abdominal cavities were examined for grossly visible lesions. Samples of larynx, trachea, lung, bronchial lymph node, and kidney were examined for lesions by light microscopy. Formalin-fixed tissues samples were embedded in paraffin, sectioned at 5 micra, and stained with hematoxylin and eosin. Some sections were stained with Masson's trichrome to delineate fibrosis. The microscopic findings in the kidney and lung were graded using the criteria described in the attached manuscript (Tables A and B, Appendix C.). A complete tabulation of the histologic findings for each rat is also shown in that Appendix (Tables C–H).

Results:

Early Deaths (previously reported and summarized here)

As previously reported, 15 of 30 rats exposed to UO_3, the most soluble of the compounds used, died or were sacrificed moribund 2–13 days after inhalation exposure (Table 1 in attached manuscript). The primary cause of death was acute tubular necrosis of the kidney involving primarily the pars recta portion of the proximal renal tubules. The tubular lining epithelium was in various stages of degeneration and necrosis. Many of the epithelial cells were necrotic and many had sloughed into the lumen (Figure 1 in attached manuscript). The acute renal tubular necrosis with death within days after exposure is consistent with renal uranium-induced toxicity previously described in rats.

All rats with renal tubular necrosis also had an acute, diffuse, suppurative pneumonia that was considered related to uremia. The pneumonia was moderate to marked in 12 of the 14 with tubular necrosis. The pulmonary lesion was characterized by widespread infiltration of neutrophilic inflammatory cells in the septa and alveoli, numerous focal proliferations of alveolar epithelial lining cells, focal accumulations of alveolar edema and focal fibroblastic organization of edema fluids (Figure 3 in attached manuscript). There was no concentration of inflammation around airways.

Zero day sacrifices (previously reported and summarized here for context)

At 4 hours after the end of inhalation exposure nephropathy was a minimal lesion seen in only 9 of 56 rats, 8 males and 1 female (Table 2 in attached manuscript) and may have represented a spontaneous renal disease often reported in rats.

Only minimal histologic changes were evident in the lungs of most of the exposed rats (Table 2 in attached manuscript).

A broncho-interstitial pneumonia was present in six of the 18 rats intranasally instilled with endotoxin 2 days before sacrifice (Figure 5 in attached manuscript) and undoubtedly a reaction to endotoxin that passed from the nasal cavity to the lung.

30-Day Sacrifices (new data)

Fifty-four rats were sacrificed 30 days after the end of inhalation exposure, as scheduled. Minimal nephropathy was present in 14 rats (Table 3, attached manuscript). It was present in 2 of the 7 air-exposed males and none of the 7 air-exposed females. A lesion classified as mild nephropathy was noted in the three male rats exposed to UO_3. The male rats exposed to the uranium compounds (UO_2, UO_3, UO_2 + UO_3, and DUO_x) had an incidence of 61% and the females 22%. This incidence was much higher than the incidence in rats exposed to air only or TaO_2: males 20% and females 0%.

A striking histologic lesion in the lung was a focal septal fibrosis that occurred in the scattered septa in the mid portion of the left lung (the only lung lobe available for examination) (Figure 6). The fibrosis consisted of focal accumulations of fibrous tissue that appear to be on the surfaces of the septa and alveolar ducts (Figure 7, attached manuscript). A few mononuclear inflammatory cells accompanied the fibrosis, but were a minor feature. The septal fibrosis occurred only in those rats exposed to the uranium compounds (Table 3, attached manuscript). Eighty to 100% of the rats exposed to UO_3 and DUO_x had minimal to mild septal fibrosis. None of the rats exposed to UO_2 and only 2 of 12 exposed to UO_2 + UO_3 had fibrosis. Males and females and those exposed to endotoxin were similarly affected.

Alveolar macrophages were increased in numbers in nearly all the rats exposed to particles. The incidence was greater than at the 0 day sacrifice. These macrophages contained particles and in greater numbers than at the 0 day sacrifices. The exception was the rats exposed to UO_3 in which not particles were seen in the alveolar macrophages.

No lesions were found in the larynx, trachea or bronchial lymph nodes.

180-Day Sacrifices (new data)

Fifty-three rats were sacrificed 180 days after the end of inhalation exposure, as scheduled (Table 4, attached manuscript). No female rats exposed to UO_3 were remaining for sacrifice at 180 days due to the large number of early deaths in this group. In addition two rats exposed to DUOx died at 135 days after exposure and one rat exposed to endotoxin was sacrificed moribund at 240 day after exposure.

A minimal nephropathy was noted in 4/18 male rats and 1/15 female rats exposed to the uranium compounds and in 2/10 males and 0/10 females exposed to air or TaO_2. This incidence in the uranium compound exposed rats (22% in males and 6.6% in females) was

much less than the incidence in uranium-compound exposed rats sacrificed at 30 days after inhalation exposure. In contrast, the incidence in the air or TaO_2 rats was similar to that in rats sacrificed at 30 days.

The septal fibrosis, first seen at the 30-day sacrifice, was still present in most of the rats exposed to UO_3 or DUOx and in one female exposed to UO_2 + UO_3. None was present in rats exposed to UO_2. The severity of the fibrosis was slightly less and accompanying mononuclear cells were minimal compared to rats sacrificed at 30 days. Hyperplasia of alveolar macrophages and the presence of particles in the macrophages were slightly less in incidence and severity compared to rats sacrificed at 30 days after exposure.

No lesions were found in the larynx, trachea or bronchial lymph nodes.

360-Day Sacrifices (new data)

Eighty-seven rats were sacrificed 360 days after the end of inhalation exposure, as scheduled (Table 5). No female rats exposed to UO_3 were remaining for sacrifice at 360 days because to the large number of early deaths in this group. In addition, one rat exposed to TaO_2 died 340 days after exposure.

A minimal to mild nephropathy was noted in 25/37 male rats and 2/30 females rats exposed to the uranium compounds and in 10/20 males and 1/20 females exposed to air or TaO_2. This incidence in the uranium compound exposed rats (68% in males and 6.6% in females) was similar to the incidence in the air or TaO_2 rats (50% in males and 5% in females) sacrificed at 360 days.

The septal fibrosis, seen at the 30- and 180-day sacrifice, was still present in most of the rats exposed to UO_3 or DUO_x and in one female exposed to UO_2 + UO_3. In addition, one male exposed to UO_2 had septal fibrosis. The severity of the fibrosis was slightly less and accompanying mononuclear cells were minimal compared to rats sacrificed at 30 days. Hyperplasia of alveolar macrophages and the presence of particles in the macrophages were reduced in incidence and severity compared to rats sacrificed at 180 days after exposure. In addition, a number of rats had minimal alveolar histiocytosis, characterized by small, focal accumulations of alveolar macrophages, typically sub-adjacent to the pleura. The air-exposed and UO_2-exposed rats had a higher incidence than rats exposed to the other compounds. Alveolar histiocytosis is a not uncommon lesion in aging rats (Montgomery and Seely, 1990b).

No lesions were found in the larynx, trachea or bronchial lymph nodes.

Summary of kidney and lung pathology from "Tank Impact Scenario" – acute, high dose

Acute renal tubular necrosis resulting in death within 14 days was found after inhalation of high concentrations of the relatively soluble uranium compound UO_3. Females were much more susceptible than males. Uremic pneumonia was a significant lesion that was also present in all the rats that died of renal toxicity. The pneumonia was severe enough to cause or contribute to the deaths. The females, with smaller lungs, may not have been able to handle the inflammation as well as the larger males, resulting in a higher incidence of deaths. Deaths due to renal toxicity did not occur with the other uranium compounds.

At 30 days after inhalation exposure, renal tubular necrosis was not present in any of the rats. However, the incidence of a lesion classified as nephropathy was greatly increased in both males and females exposed to the uranium compounds when compared with the air or TaO_2 exposed rats where no renal toxicity would be expected (Table 6, attached manuscript). Much of the lesion in the uranium-exposed rats was probably related to repair of the tubules following the acute renal tubular necrosis, which could not be differentiated histologically from the spontaneous lesion of nephropathy. The incidence of nephropathy at 180 and 360 days after exposure is essentially the same as the air and TaO_2-exposed rats, which can be considered the background for spontaneous nephropathy. This indicates that a non-fatal acute tubular necrosis probably occurred in about 40% of the males and 20% of the females exposed to the uranium compounds, including DUO_x.

The pulmonary septal fibrosis seen 30 days after exposure in the rats exposed to several of the uranium compounds is a novel finding (Table 7, attached mansucript). Two unusual features of the fibrosis were the focal nature of the lesion and the distribution in the central portion, not the cranial or caudal portions of the left lung. The lesion was seen in 100% of the rats surviving the UO_3 exposure and in 67% to 100% of the rats exposed to DUO_x. It was not seen in any of the air or TaO_2-exposed rats.

The septal fibrosis is considered to be sequelae of uremic pneumonia. This is based on the indication that rats exposed to UO_3, the group that had the highest incidence and severity of fibrosis, was also the group that had rats dying with uremic pneumonia. It is plausible to think that the surviving rats also had a uremic pneumonia. In addition, the distribution of the fibrosis in the lung is unusual. There are no available reports on the distribution or sequelae of uremic pneumonia in rats. However, the distribution of fibrosis is not similar to that of inhaled particles (Dungworth et al., 1995 as cited in attached manuscript). Such fibrosis is centered on the distal airways, is more uniform in distribution within the lung and is usually involves the interstitium to some degree.

These data indicate that the acute, high dose inhalation exposures used in this study to simulate exposure to individuals in the proximity of a direct tank hit could produce kidney toxicity, resulting in long-lasting fibrotic changes in lung. Because females were more susceptible to the acute toxicity resulting from the initial exposure, no females remained in the later sacrifice times to evaluate whether or not there is increased sensitivity in females to the long-term sequelae of these exposures. The fibrosis secondary to uremic pneumonia was associated with the form of the uranium compounds, as it was observed in virtually all animals exposed to either UO_3 or to DUOx, but only rarely following exposure to UO_2+UO_3. and in only 1 animal exposed to UO_2. The consistency of response between UO_3 and DUOx with only minimal response to the UO_2+UO_3 mixture suggests more than solubility determined the response. We are following up on this now. In addition, a formal statistical analysis of the data with respect to the time course of response will be completed prior to submission of the attached draft for publication.

B. "March Through Scenario": Short duration, moderate exposure time

TASK 1: ***Sacrifices of rats exposed via nose-only inhalation for 8 hours to aerosols of a mixture containing by weight 50% UO_2 and 50% UO_3 at a dose of 1 mg/m^3 will be performed. A separate air-only exposed control group will be employed as vehicle control. For each group rats will be sacrificed in sets of 6 per group immediately following exposure, and at 30, 180 and 360 days post-exposure. Tissue analyses for longer duration exposures will begin in Year 2, but substantially carry over to Year 3***

Note: Animals were sacrificed at 0, 30 and 180 days post-exposure. Rats originally designated to a 360 day post-exposure sacrifice group, as well as remaining spare animals, were re-exposed as described below under "E. Re-exposure scenario"

Methods:

Animal sacrifices and tissue collection
At day of completed exposure (within 2 h – 0 day sacrifice) as well as 30 days post-exposure rats were sacrificed by CO_2 inhalation and exsanguination by cardiac saline perfusion. The brain was removed and frozen in dry ice-cooled isopentane at –42°C and transferred at the end of the day to –80°C for long-term storage. Spinal cords were removed and immersion fixed in 4% paraformaldehyde. At 180 days post-exposure, rats were sacrificed by CO_2 inhalation and exsanguination by cardiac saline perfusion followed by perfusion with 4% paraformaldehyde. The brain and spinal cord were removed and post-fixed in 4% paraformaldehyde.

For all rats (0, 30 and 180 days post-exposure) the nose, with skin and lower jaw removed, was fixed in 4% paraformaldehyde. The left and right lungs were weighed. The left lung was perfused with 4% paraformaldehyde and the right was frozen. The larynx, trachea and bronchial lymph node were fixed in 4% paraformaldehyde. The left and right kidneys were weighed. The left kidney was fixed in 4% paraformaldehyde and the right was frozen. Both femurs were weighed and frozen for chemical analysis.

Results:

Exposure and sacrifices
Sacrifices at 30 and 180 days post-exposure were completed in Year 3 (0 day sacrifices completed in Year 2).

Bodyweight:
Both male and female rats from all exposure groups gradually gained weight over time (Fig. 1), with males showing more rapid and greater total gain. Detailed statistical analysis is in progress.

Brain weight:

Preliminary statistical analysis revealed an expected effect of gender ($p<0.0001$) and time post-exposure ($p=0.004$) (3 factor ANOVA), but no effect of exposure group ($p=0.156$). There was no significant interaction between the three factors.

TASK 2: ***Quantitation of metals in nose and brain tissues will be performed with Atomic Absorption Spectrophotometry (AAS) and microbeam Proton Induced X-ray Emission (μ-PIXE)***

Methods:

Tissue sectioning and preparation

Frozen tissue sections were cut at 10 μm using a Hacker-Bright cryostat. Three levels of sagittal sections containing the brain regions of interest were sampled. Anatomical regions within each of the levels are summarized in Table 2. One section from each level was mounted on a nylon foil and freeze-dried for PIXE analysis. One section from the same level was stained with hematoxylin and eosin, digitally scanned, and regions for PIXE analysis were outlined to ensure beam localization within correct brain regions (Figure 1). Additional sections from each level were retained for future immunohistochemical analysis.

Table 2. Brain regions within each sampled sagittal level of rat brain

Sectioning level	Anatomic Structure	Lateral distance from midline
1	Caudate putamen Globus Pallidus Substantia nigra	~2.9 mm
2	Caudate putamen Substantia nigra	~1.9 mm
3	Glomeruli Mitral cell layer Olfactory Tuberculum Substantia nigra	~0.9 mm

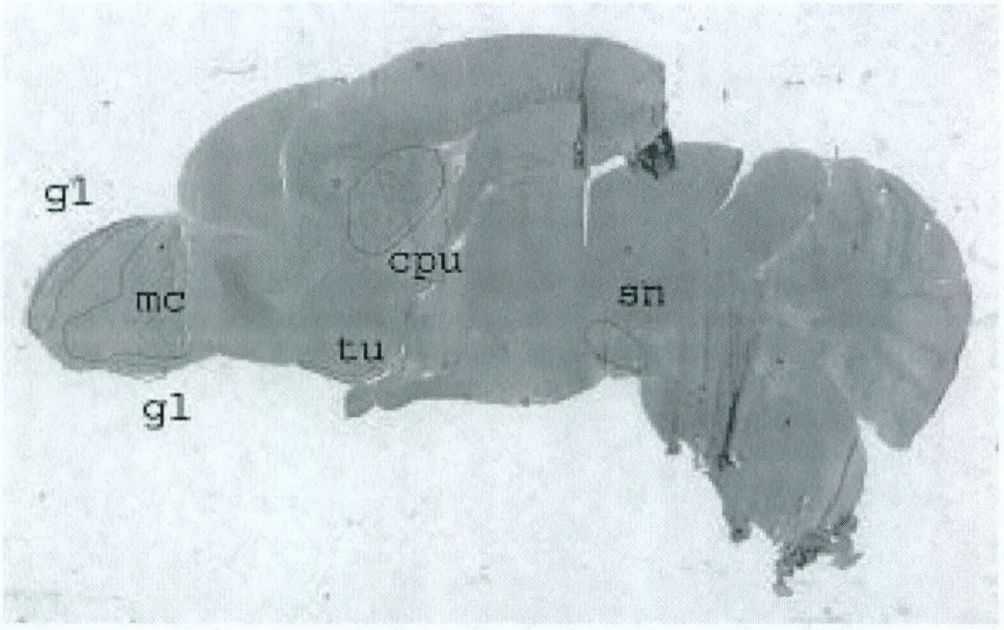


Figure 1. Hematoxylin and eosin stained sagittal brain section with anatomic structures of interest outlined where: gl=glomeruli; mc=mitral cells; tu=tuberculum; cpu=caudate putamen; sn=substantia nigra.

PIXE measurement of metal content

Uranium concentrations in localized brain regions (glomeruli, mitral cells, olfactory tuberculum, caudate putamen, and substantia nigra) were determined with Proton Induced X-ray Emission (PIXE). PIXE is an x-ray fluorescence technique that uses MeV energy proton beams to interrogate specimens. It provides accurate quantitation, simultaneous multi-element detection and is capable of micron-scale spatial resolution whilst maintaining down to 1 mg/g elemental sensitivity. Regions of interest within the freeze-dried tissue sections were identified visually using stained adjacent serial sections. Regions of interest were irradiated with 3 MeV proton microbeams for doses of up to 15 micro coulombs. Beam spot sizes were typically between 0.3x0.3 and 0.5x0.5 mm. X-ray yields for uranium were monitored using an energy dispersive x-ray detector. Yields were converted to quantitative concentrations using thin film standards of uranium of known thickness to determine detector efficiency. The system has been tested on certified standards and has quantitative accuracy of better than 95% for analysis of metals in biological matrices.

Results:

At the 0 day post-exposure time point, one female animal exposed to UO_2+UO_3+endotoxin displayed a uranium concentration of 3.3 ± 1.8 mg/kg in the glomeruli and 3.0 ± 1.6 in the mitral cell layer. One male UO_2+UO_3 exposed animal analyzed at the same timepoint exhibited a glomerular uranium concentration of 3.0 ± 1.6 mg/kg. Uranium levels for all other rats analyzed 0 days post-exposure (in total 6 uranium oxide and 6 uranium oxide + endotoxin-exposed rats), as well as other brain structures for the 2 rats with detectable glomerular uranium levels, were below the detectable level (MDL) of 2.1-2.9 mg/kg. Due to the low concentration observed in those animals with detectable uranium, the low frequency of detection, and our previous information on the rate of decrease in other transported brain metals with time and deeper tissue transport, later time-points (30 and 180 days) were not analyzed for uranium content. Rather, the 180 day tissues were perfused and fixed in paraformaldehyde for neurochemical analyses. These analyses will be targeted in the coming year to supplement data from the C and E exposures.

C. "Clean-up Scenario 1": Long duration, moderate exposure time without endotoxin

TASK 1: ***Sacrifices of rats exposed via nose-only inhalation for 30 days to aerosols of a mixture containing by weight 50% UO_2 and 50% UO_3 at a dose of 1 mg/m^3 will be performed. A separate air-only exposed control group will be employed as vehicle control. For each group rats will be sacrificed in sets of 6 per group immediately following exposure, and at 30, 180 and 360 days post-exposure. Tissue analyses for longer duration exposures will begin in Year 2, but substantially carry over to Year 3***

Note: Animals originally designated to a 360-day post-exposure sacrifice group, as well as remaining spare animals, were re-exposed as described below under "E. Re-exposure scenario".

Methods:

Animal sacrifices and tissue collection
All sacrifice and tissue retrieval methods were identical to those described above for the "March Through" scenario.

Results:

Exposure and sacrifices
Sacrifices at 30 and 180 days post-exposure were completed in Year 3 (0 day sacrifices were completed in Year 2).

Bodyweight:
Rats did not gain weight during the duration of exposure (when animals were restrained in nose-only inhalation tubes for 6h/day, 5 days/week). This weight-plateau was similar for all exposure groups. After the end of exposures, bodyweight gradually increased for all experimental groups (Fig 2). Again, males showed more rapid and higher maximum weight gain. Based on preliminary analysis, exposure condition does not appear to affect bodyweight. Detailed statistical analysis is in progress.

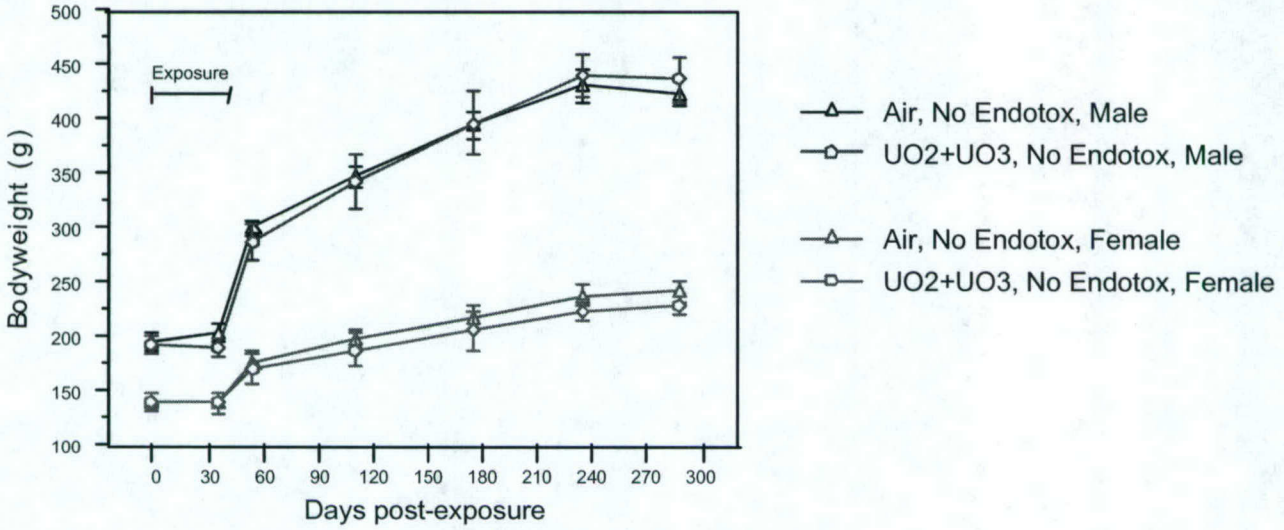


Figure 2: Bodyweight of rats exposed for **30 days** to Air or 1mg/m^3 UO_2+UO_3 in summer 2003. Graphs represent mean +/- SD.

Brain weight:

Preliminary statistical analysis revealed a significant effect of gender ($p<0.0001$) but no time post-exposure ($p=0.248$) or exposure effect ($p=0.526$) (3 factor ANOVA). There was no significant interaction between the three factors (Figure 3).

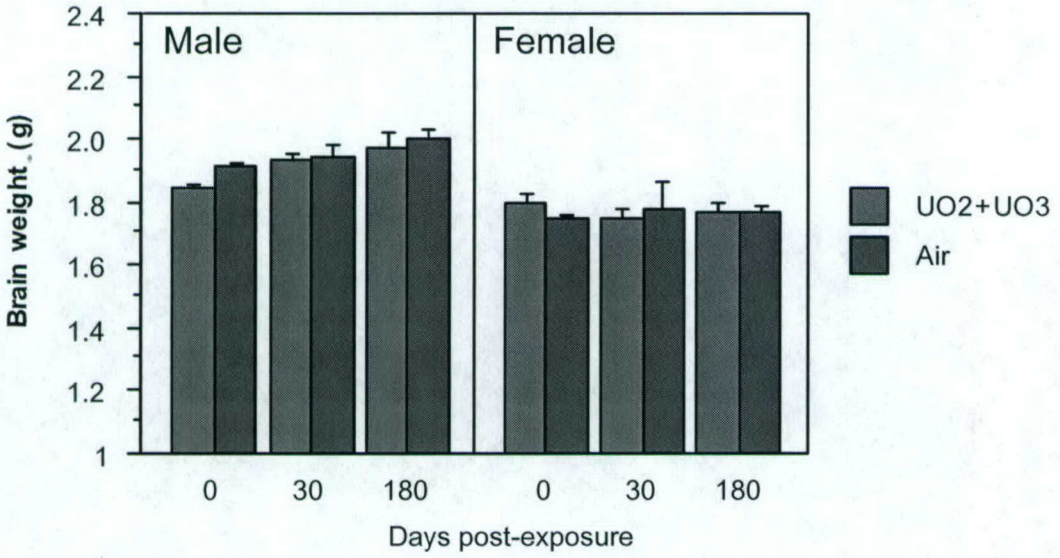


Figure 3: Brain weights from male (left) and female (right) rats exposed for **30 days** to Air or 1mg/m^3 UO_2 + UO_3. Bars represent mean +/- SEM.

TASK 2: ***Quantitation of metals in nose and brain tissues will be performed with Atomic Absorption Spectrophotometry (AAS) and microbeam Proton Induced X-ray Emission (μ-PIXE)***

Methods: See "March-Through" scenario exposure above.

Results:
Uranium levels for all rats and all structures analyzed 0 days post-exposure (in total 6 uranium oxide-exposed rats) were below the detectable level (MDL) of 2.0-3.0 mg/kg. Due to the low frequency of detection, later time-points (30 and 180 days) were not analyzed for uranium content.

TASK 3: ***Examining the spinal cords in the exposed animals for markers of damage associated with amyotrophic lateral sclerosis, a neurodegenerative disease recently noted to occur at increased prevalence in Gulf War veterans. (NOTE: this task was added during the approved rescoping that occurred in 2004. In year 3, pilot analyses were begun on animals already exposed, and more systematic investigation of this pathology will occur in conjunction with the final exposure described in E.)***

Methods:
Lumbar and cervical spinal cord enlargement was sectioned at 10μm thickness on a cryostat. Serial sections 200 μm apart (approximately 10 sections per region) were mounted on glass slides and stained with Cresyl Violet to visualize neurons. The large (>25μm in diameter) spinal motor neurons in the ventral half of the spinal cord were easily visualized and counted under light microscope. The main pathological feature in transgenic mouse models of amyotrophic lateral sclerosis is a loss of lumbar large spinal motor neurons (e.g. Karlsson et al. 2004). The mean number of large motor neurons per section in the different experimental groups was compared.

Results:
Preliminary results show no significant difference in number of lumbar large motor neurons between uranium oxide and air-exposed animals.

Table 3. Number of large lumbar spinal motor neurons per section

Exposure	Gender	Days PE	n	Number of neurons ± SD
Air	M	0	2	8.6 ± 2.0
UO2+UO3	M	0	2	9.9 ± 3.3
Air	F	0	2	7.7 ± 0.6
UO2+UO3	F	0	2	6.1 ± 0.7
Air	M	30	3	7.8 ± 2.4
UO2+UO3	M	30	3	8.0 ± 0.8
Air	F	30	3	6.7 ± 0.5
UO2+UO3	F	30	3	6.9 ± 1.1

Evaluation of cervical motor neurons was difficult due to vacuolization of neurons and hence difficulties in determining neuron size accurately. The vacuolization was present in both air-

and uranium-exposed animals and is most likely due to artifacts at tissue retrieval. Since brains from the same animals were to be used for PIXE analysis, rats could not be perfused with paraformaldehyde. Instead, spinal cords were immersion fixed. In the 30 day uranium oxide exposures initiated during year 3, a subset of the exposed animals were perfused with paraformaldehyde at the 0 day sacrifice timepoint in order to ensure a more rapid fixation of tissue (see below).

This investigation was a pilot attempt to test procedures on frozen tissue. The difficulties encountered informed the proposed methods in the rescoping and the more complete analysis will therefore be completed on perfused tissues form the final exposures described in E.

RESCOPING APPROVED IN 2004:

D. "Clean-up Scenario 2": Long duration, short time, with or without endotoxin

TASK 1: ***Completing endotoxin exposure in Clean-up Scenario originally proposed and replacing the Maintenance Scenario with additional animals in the "Clean-up Scenario" exposures to clarify and expand positive findings seen to date.***

To probe some of the findings in the earlier analyses, the rescoping adding the following analytical components performed by additional collaborators as noted:

- Improved detection limit analysis of U in kidney, blood, and lung using ICP-MS. These analyses will be performed in Dr. Donald Smith's laboratory at the University of California, Santa Cruz by Dr. Robert Gwiazda. Dr. Gwiazda has performed and published on these analyses previously.
- Oxidative damage, mitochondrial damage, DNA damage and repair in brain and thoracic spinal cord. These analyses will be performed by Dr. Fernando Cardozo-Pelaez at the University of Montana who has published on these methods in conjunction with exposures to other metals.
- Addition of more detailed analysis of spinal cord damage as related to markers of Amyotrophic Lateral Sclerosis to be performed by Dr. Jenny Karlsson in the UNM laboratory.

Methods:

Animals

A total of 139 (68 male and 71 female) Fischer 344 rats, 9-10 weeks old, (Harlan Sprague Dawley, Indianapolis, IN) were used. All rats were quarantined for 10 days, housed 2 to 3 per cage in shoebox cages with hardwood chip bedding. They were fed Teklad Certified Rodent Diet (W). Food and water were available *ad libitum* except during exposure. The animal rooms were maintained at 20-22°C and 30-70 % relative humidity. A 12-hour, light/dark cycle, was maintained with lights on at 0600. The rats were randomized by weight into exposure groups as outlined in Table 4. Rats were identified by tail tattoo using an alpha numeric numbering system.

Endotoxin Instillation

To induce inflammation in the nasal mucosa, two groups of rats (Table 4) were intranasally instilled with endotoxin (Sigma Chemical Co., St. Louis, MO, Lipopolysaccharide from Pseudomonas aerugenosa Serotype 10, 1 mg/ml) following the procedures of Harkema and Hotchkiss (1991). Rats were anesthetized by halothane inhalation, removed in a light plane of anesthesia, and instilled with endotoxin (200 µg total) by placing 2 - 50 µl drops in each nostril. Endotoxin instillations were made weekly (every Saturday), 48 hours before start of each week's 5-day exposure session (Monday-Friday) to uranium aerosols or air.

Table 4. Experimental groups for moderate dose (1 mg/m^3 UO_2+UO_3) 30day exposure

EXPOSURE		0 days		14 days		30 days		180 days		Extra	
Condition	*Duration*	*M*	*F*	*M*	*F*	*M*	*F*	*M*	*F*	*M*	*F*
UO_2+UO_3	30 day	6	6	3	3	3	3	3	3	1	2
UO_2+UO_3 + Endotoxin	30 day	6	6	3	3	3	3	3	3	1	2
Air	30 day	6	6	3	3	3	3	3	3	1	1
Air + Endotoxin	30 day	6	6	3	3	3	3	3	3	1	1
No exposure	N/A	2	2	2	3						

M=male, F=female

Generation of UO_2 + UO_3 aerosols

Uranium Oxides were purchased from CERAC, Inc. P.O. Box 1178, Milwaukee, WI 53201-1178 (UO2 - CAS# 1344-57-6 50 mesh, 99.8% purity and UO3 - CAS# 1344-58-7 Powder, 99.8% purity). Aerosol of the UO2 + UO3 mixture was generated using a Wright Dust Feeder (BGI, Inc,). All stock materials required prior ball milling to approximately 5 mm and sieving to achieve a material size suitable for aerosol generation. Aerosols generated by Wright Dust Feeder (WDF) were diluted with clean, filtered air and passed through a cyclone to remove the fraction of aerosol larger than approximately 5 microns. The aerosol was then fed into a 96-port nose-only exposure system that was operated at a flow rate of approximately 30 L/min. The WDF speed was adjusted to deliver the target concentration to the exposure chamber. These procedures follow Standard Operating Procedures used within LRRI (Dunnick et al., 1988; Raabe et al., 1973). Prior to beginning actual exposures, all aerosols were tested in the exposure system to ensure target concentrations were achievable, could be reliably generated, and maintained for the necessary duration. Also, aerosols were evaluated for particle size and chemical purity.

Inhalation exposures

Before exposure, rats were conditioned to nose-only restraint tubes for at least two periods, the first for ~20 minutes and the second, conducted on a separate day, for ~40 minutes. Rats were exposed nose-only for 30 days (6 h/day, 5 days/week for 6 weeks) to 1.0 mg/m^3 of UO_2 + UO_3 mixture or to clean-filtered air only. See Table 4 for details.

Animal sacrifices and tissue collection

Three male and three female animals per exposed group were sacrificed and perfused with saline at each of the following time-points: 0-4h post-exposure (0 day), 14 days or 30 days post-exposure. In addition, 2 males and 3 females from the un-exposed group were sacrificed and saline-perfused 14 days post-exposure. A summary of retrieved tissues and planned analysis is outlined in Table 5. After CO_2 inhalation, 2-4ml blood were collected from the heart and frozen at –20°C. Left kidney was tied off, removed, weighed and frozen at –20°C. After intracardial saline perfusion, the brain was removed and weighed. One brain hemisphere was frozen in dry-ice-cooled isopentane at –42°C, and the other hemisphere was frozen in liquid nitrogen. Brain tissue was transferred to –80°C for long-term storage. Cervical and lumbar spinal cord was dissected, post-fixed in 4% paraformaldehyde and then transferred to 20% sucrose for storage. Thoracic spinal cord was dissected, frozen in liquid nitrogen and stored at –80°C. The nose, with skin and lower jaw removed was fixed in 4% paraformaldehyde. The left and right lungs were weighed. The left lung was perfused with 4% paraformaldehyde

and the right was frozen at –20°C. The larynx, trachea and bronchial lymph node were fixed in 4% paraformaldehyde. The right kidney was weighed and fixed in 4% paraformaldehyde. The carcass was weighed and frozen for chemical analysis.

Three male and three female animals from each exposed group and 2 male and 2 female unexposed animals were perfused with saline followed by 4% paraformaldehyde on the last day of exposure (0 day). Brain and spinal cord were dissected, post-fixed in 4% paraformaledhyde for 24h and then transferred to 20% sucrose for storage. Non-CNS tissue was collected exactly as described above for saline-only perused animals.

Table 5. Tissue retrieval and planned analysis for moderate dose (1 mg/m^3 UO_2+UO_3) 30day exposure

Tissue	Preservative	Analysis (Facility)
Saline only perfused animals (0, 14 and 30 days post-exposure)		
1/2 Brain	Freeze –42°C isopentane	Histology (UNM), Uranium uptake (LLNL)
1/2 Brain	Freeze liquid nitrogen	Neurochemistry (U Montana)
Spinal cord (cervical and lumbar)	4% PFA 24 h	Histology (UNM)
Spinal cord (thoracic)	Freeze liquid nitrogen	Neurochemistry (U Montana)
Saline+paraform perfused animals (0 and 180 days post-exposure)		
Brain	4% PFA 24 h	Histology (UNM)
Spinal cord (cervical and lumbar)	4% PFA 24 h	Histology (UNM)
All animals (0, 14, 30 and 180 days post-exposure)		
Blood (heart)	Freeze –20°C	Uranium content (U Santa Cruz)
Nose	4% Paraform.	Histology (LRRI)
Lung - left	4% Paraform	Histology (LRRI)
Lung – right	Freeze –20°C	Uranium content (U Santa Cruz)
Larynx	4% Paraform	Histology (LRRI)
Trachea	4% Paraform	Histology (LRRI)
LN, Bronchial	4% Paraform	Histology (LRRI)
Kidney – left	4%Paraform	Histology (LRRI)
Kidney – right	Freeze –20°C	Uranium content (U Santa Cruz)
Carcass	Freeze –20°C	Uranium content (LLNL)

Results:

Exposure Atmosphere Characterization

The UO_2 + UO_3 concentration in the aerosol was 1.02 ± 0.04 mg/m^3, size distribution was 1.74 ± 0.16 μm with a sigma-g of 1.57 ± 0.12. Target concentration was 1 mg/ m^3. PIXE analysis of filters from test ports in the incubation chambers confirmed only uranium to be present in each filter. Minimum detection limits for other elements were ~ 0.1 μg/g.

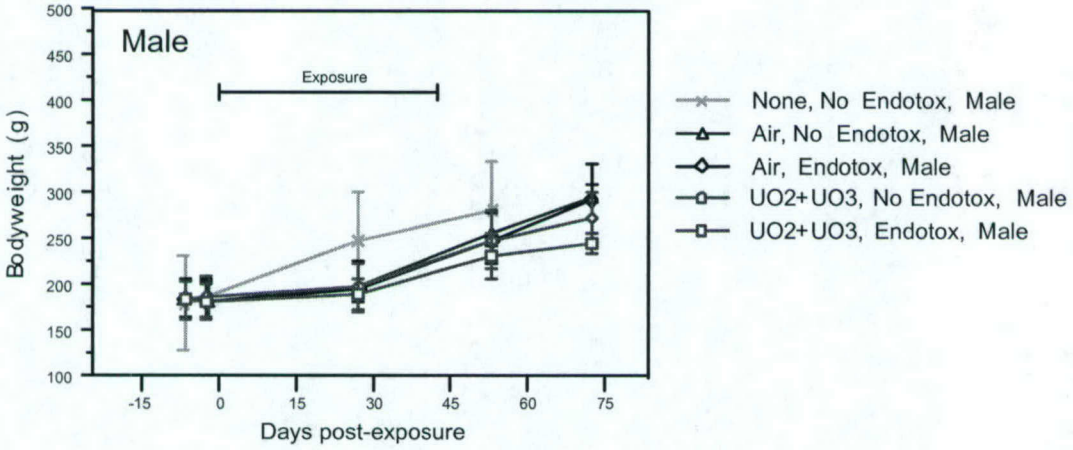
Exposure and sacrifices

Animal exposures have been completed during the weeks of July 26th – September 3rd. Sacrifices at 0, 14 and 30 days post-exposure have been completed in October 2004. The last sacrifice (180 days post-exposure) is planned for March 2005.

Body weight

Bodyweights have been measured up to 30 days post-exposure (Fig 4A and 4B). As expected, female rats weighed less than male. The bodyweight of unexposed rats (that have not been subjected to restraint in inhalation tubes) gradually increased during the 2 month period. In contrast, rats exposed to air or uranium oxide did not gain weight as fast. In preliminary analysis, neither exposure condition nor endotoxin appear to affect bodyweight. Detailed statistical analysis is in progress.

A



B

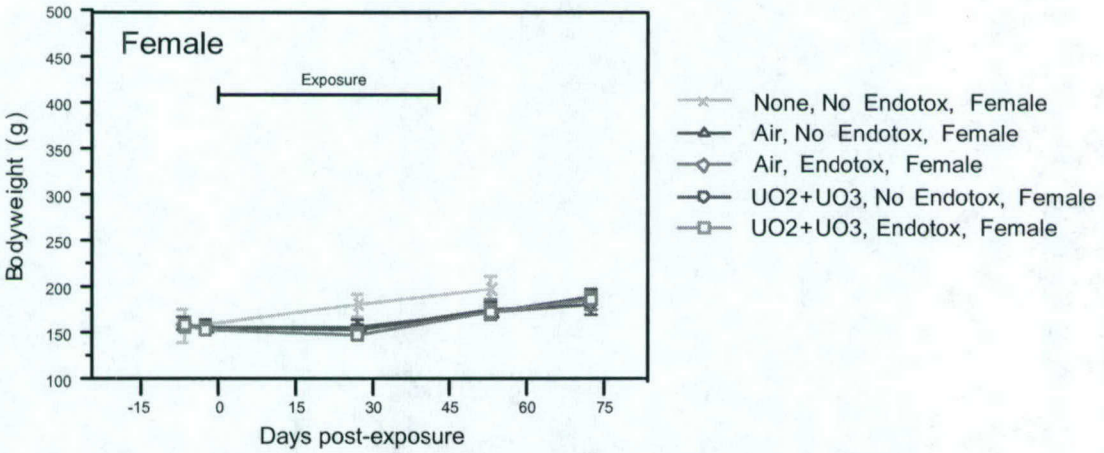


Figure 4: Bodyweight of male **(A)** and female **(B)** rats exposed for **30 days** to Air or 1mg/m^3 UO_2+UO_3, with or without weekly nasal endotoxin. Graphs represent mean +/- SD.

Brain Weights:

Brain weights have been obtained from the sacrificed animals (up to 30 days post-exposure)(Fig 5). Preliminary statistical analysis revealed an expected effect of gender ($p<0.0001$) and time post-exposure ($p<0.0001$), but no exposure effect ($p=0.583$) or endotoxin effect ($p=0.672$) (4 factor ANOVA).

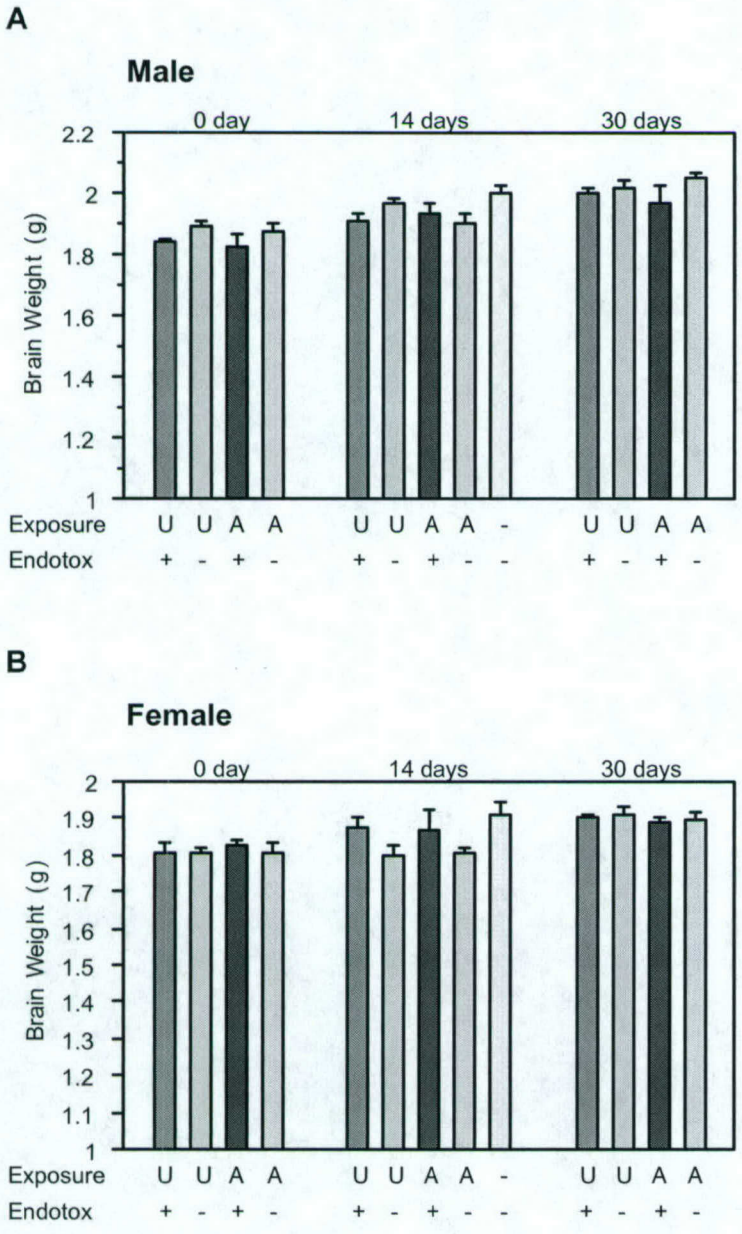


Figure 5: Weight of brains from male **(A)** and female **(B)** rats exposed for **30 days** to Air or 1mg/m^3 UO_2+UO_3, with or without weekly nasal endotoxin, Animals were sacrificed 0, 14 or 30 days post-exposure. Bars represent mean +/- SEM.

TASK 2: ***Examining the spinal cords in the exposed animals for markers of damage associated with amyotrophic lateral sclerosis, a neurodegenerative disease recently noted to occur at increased prevalence in Gulf War veterans.***

In the 30 day uranium oxide exposures initiated during year 3, a subset of the exposed animals were perfused with paraformaldehyde at the 0 day sacrifice timepoint. In addition, unexposed rats (that had not been restrained in inhalation tubes) were introduced as an additional control group. To date, we have started sectioning the spinal cords and fine-tune the immunohistochemical protocols. At this initial stage, we do not see the pattern of intracellular vacuoles as compared to the previous exposure (see C. Clean-up scenario 1"). We have successfully set up a protocol for immunohistochemical staining of microglia in these tissues (Figure 6). ALS transgenic rats from other ongoing studies in our laboratory were used as a positive control for neuroinflammation. Evaluation of the 0 day post-exposure sacrifice spinal cords is currently ongoing.

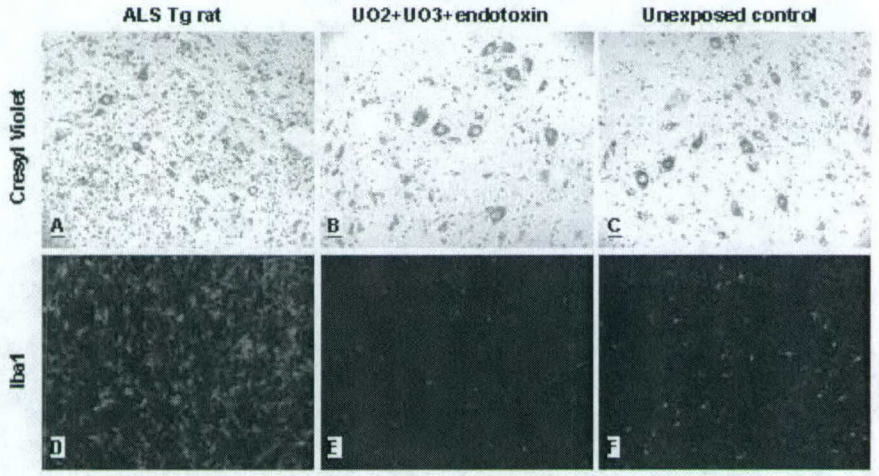


Figure 6. Lumbar spinal cord sections from an ALS transgenic rat (A, D), UO_2+UO_3+endotoxin (B, E), and unexposed control rat (C, F). Sections were either stained for Cresyl Violet (A-C) or immunohistochemically stained for Iba1 to detect microglia. Note the loss of large spinal motorneurons and the extensive inflammation in the positive control ALS rat.

Ongoing analysis

Blood, kidney and lung tissue to be analyzed for uranium content has been sent to University of Santa Cruz.

Brain and thoracic spinal cord tissue to be analyzed for neurochemical markers of cell damage has been sent to University of Montana. DNA damage, oxidative stress and mitochondrial damage will be analyzed in homogenates from sub-dissected brains and spinal cord.

Tissue analysis will be performed predominately in Year 4 but for the later time-points be extended in to Year 5. We are anticipating a the need for a no-cost extension to the initial period of performance in order to complete all outstanding analyses.

E. "Re-exposure" scenario

TASK 1: ***Re-expose of animals previously exposed and held for long-term survival.***

Methods:

Animals

A total of 41 rats from the 2003 exposures, originally intended as a 360-day post-exposure analysis group, were selected for re-exposure. Animals were assigned to UO_3+UO_3+endotoxin or air+endotoxin re-exposure groups as outlined in Table 6. Twelve additional rats exposed to air+endotoxin for 1 day in 2003 were NOT re-exposed but sacrificed at the same time as re-exposed animals as a comparison for neurochemical changes resulting from age alone. Between the first exposure and the re-exposure, animals were housed at standard conditions and weighed monthly.

Table 6. Re-exposure (1 mg U/m^3) of previously exposed (1 mg U/m^3) rats

PREVIOUS EXPOSURE		RE-EXPOSURE		0 day sacrifice
Condition	*Duration*	*Condition*	*Duration*	*N*
Air	30 day	UO_2+UO_3 + Endotoxin	30 day	4
		Air + Endotoxin	30 day	4
UO_2+UO_3	30 day	UO_2+UO_3 + Endotoxin	30 day	6
		Air + Endotoxin	30 day	4
UO_2+UO_3	1 day	UO_2+UO_3 + Endotoxin	30 day	7
		Air + Endotoxin	30 day	4
UO_2+UO_3 + Endotoxin	1 day	UO_2+UO_3 + Endotoxin	30 day	8
		Air + Endotoxin	30 day	4
Air +Endotoxin	1 day	No exposure	N/A	12

Previous exposure date: 10/1/2003 (1 day exposed animals), 8/25-10/6/2003 (30 day exposed)

Endotoxin instillation and inhalation exposures.

Procedures for endotoxin instillation, animal conditioning and exposure atmosphere generation were as described above for "Clean-up scenario 2". Briefly, rats were re-exposed to 1.0 mg/m^3 of UO_2+UO_3 mixture or to clean-filtered air for 30 days (6 h/day, 5 days/week for 6 weeks). All re-exposed rats received weekly endotoxin instillations 48 h prior to the weekly exposure session. Finally, 6 male and 6 female rats initially exposed to Air+Endotoxin for 1 day (see Table 6) were not re-exposed but sacrificed at the same time as re-exposed rats.

Animal sacrifices and tissue collection

All rats were sacrificed at day of completed exposure (0-6h after exposure) and were perfused with saline. Euthanasia and tissue retrieval was identical to procedures described for saline-only perfused rats in "Clean-up scenario 2" above. A summary of retrieved tissues is outlined in Table 7. As these reexposures were part of the rescoping, the additional analyses described in Section D above are also being done on tissues from the reexposed animals.

Table 7. Tissue retrieval and planned analysis for re-exposed animals

Tissue	Preservative	Analysis (Facility)
1/2 Brain	Freeze –42°C isopentane	Histology (UNM), Uranium uptake (LLNL)
1/2 Brain	Freeze liquid nitrogen	Neurochemistry (U Montana)
Spinal cord (cervical and lumbar)	4% PFA 24 h	Histology (UNM)
Spinal cord (thoracic)	Freeze liquid nitrogen	Neurochemistry (U Montana)
Blood (heart)	Freeze –20°C	Uranium content (U Santa Cruz)
Nose	4% Paraform.	Histology (LRRI)
Lung - left	4% Paraform	Histology (LRRI)
Lung – right	Freeze –20°C	Uranium content (U Santa Cruz)
Larynx	4% Paraform	Histology (LRRI)
Trachea	4% Paraform	Histology (LRRI)
LN, Bronchial	4% Paraform	Histology (LRRI)
Kidney – left	4%Paraform	Histology (LRRI)
Kidney – right	Freeze –20°C	Uranium content (U Santa Cruz)
Carcass	Freeze –20°C	Uranium content (LLNL)

Results:

Exposure Atmosphere Characterization
The UO_2 + UO_3 concentration in the aerosol was 1.02 ± 0.04 mg/m^3, size distribution was 1.74 ± 0.16 μm with a sigma-g of 1.57 ± 0.12. Target concentration was 1 mg/m^3. PIXE analysis of filters from the incubation chambers confirmed only uranium to be present in each filter. Minimum detection limits for other elements were ~ 0.1 μg/g.

Exposure and sacrifices
Animal re-exposures have been completed during the weeks of July 19^h – Aug 27th 2004. All rats were sacrificed the same day as the last exposure (0 day timepoint).

Bodyweight

A described above for "Clean-up" scenarios, bodyweight did not increase during the initial 30 day exposure period. After completing the initial exposure, weight gain was consistent in all groups until animals were re-exposed. Bodyweight then decreased in re-exposed animals whereas rats not subjected to re-exposure maintained their weight. Preliminary analysis indicates re-exposure condition does not appear to affect bodyweight. Detailed statistical analysis is in progress.

Brain weight

Statistical evaluation is in progress, but preliminary analysis indicates no consistent effect of exposure conditions on brain weight.

TASK 2: ***Quantitation of metals in nose and brain tissues will be performed with Atomic Absorption Spectrophotometry (AAS) and microbeam Proton Induced X-ray Emission (PIXE)***

Methods: See "March-Through" scenario exposure above.

Results:
To date, brains from the rats exposed to UO_2+UO_3 for 30 days in summer of 2003 and then re-exposed to UO_2+UO_3 +endotoxin or air+endotoxin for 30 days in summer 2004 have been analyzed for uranium content. Uranium deposition was ONLY seen in glomeruli in one animal (male exposed to UO_2+UO_3+endotoxin). Detected uranium concentration was 3.8 ± 2.2 mg/kg. MDL for glomeruli in this animal was 2.6 mg/kg. No measurable levels of uranium were detected in other animals or in other brain structures in the animal showing uranium deposition in glomeruli. Table 8 shows uranium MDL and sample size for the re-exposed animals.

Table 8. Uranium MDL ± SD (mg/kg) in re-exposed animals

Exposure	Sex	n	Glomeruli	Mitral Cells	Olfactory Tuberculum	Caudate Putamen	Globus Pallidus	Substantia Nigra
Air	M	1	2.7	2.4	2.7	2.3	2.1	2.7
Air	F	1	3.0	2.8	2.7	2.0	2.7	2.4
UO_2+UO_3	M	4	2.8 ± 0.9	2.7 ± 0.7	2.9 ± 0.1	2.6 ± 0.6	2.6 ± 0.7	2.6 ± 0.9
UO_2+UO_3	F	2	2.5 ± 0.5	2.6 ± 0.4	2.5 ± 0.5	2.6 ± 0.1	2.5 ± 0.1	2.7 ± 0.15

All animals exposed to 30 days UO_2+UO_3 summer 2003. Exposed to 30 days UO_2+UO_3 + Endotoxin *or* 30 days Air + Endotoxin summer 2004. Analysis of brains sacrificed at last day of exposure (0 day sac point).

Ongoing analysis:

Blood, kidney and lung tissue to be analyzed for uranium content has been sent to University of Santa Cruz.

Brain and thoracic spinal cord tissue to be analyzed for neurochemical markers of cell damage has been sent to University of Montana. DNA damage, oxidative stress and mitochondrial damage will be analyzed in homogenates from sub-dissected brains and spinal cord.

Remaining tissue analysis will be performed in Year 4 and is anticipated to require a no-cost extension of the period of performance for completion in the following year.

KEY RESEARCH ACCOMPLISHMENTS DURING YEAR 3

Tank impact scenario: *Acute, high dose exposure (begun in Year 1)*

- Full analysis of lung- and kidney pathology from short-term exposed animals indicates long-lasting septal fibrosis in lung resulting from uremic pneumonia following acute, high-dose inhalation of either soluble UO_3 or Depleted Uranium oxide.
- Analysis of kidney uranium content from early death acute UO_3-exposed animals.

March through scenario: *Moderate dose, short-term exposures (initialized in Year 2)*

- Completion of sacrifices (0, 30 and 180 days post-exposure)
- Initial analysis of body and brain weight
- Analysis of uranium content in several brain regions from animals sacrificed 2 h (0 day) post-exposure indicates first documented uptake of uranium in glomeruli and deeper mitral cells.

Clean-up scenario 1: *Moderate dose, long-term exposures (initialized in Year 2)*

- Completion of sacrifices (0, 30 and 180 days post-exposue)
- Initial analysis of body and brain weight
- Analysis of uranium content in several brain regions from animals sacrificed 2 h (0 day) post-exposure.
- Quantification of large spinal motor neurons from 30 day exposed animals sacrificed 2 h (0 day) and 30 days post-exposure.

Clean-up scenario 2:– *additional animals (initialized in Year 3)*

- Completion of moderate-dose 30day exposures with or without endotoxin.
- Completion of the 0, 14 and 30 day post-exposure sacrifices.
- Initial analysis of body and brain weight

Re-exposure scenario: *Moderate dose, long-term exposures of moderate dose exposed animals from Year 2 (initialized in Year 3)*

- Completion of moderate-dose 30day re-exposure of animals
- Sacrifice of all re-exposed animals.
- Initial analysis of body and brain weight
- Initial analysis of brain uranium content in re-exposed animals again demonstrates uptake in one animal.

Initiation of research collaborations: As described previously, we have initiated a collaboration with Dr Fernando Cardozo-Pelaez at University of Montana. Dr Cardozo-Pelaez will analyze brain and spinal cord from rats exposed during summer of 2004. In addition, Dr Roberto Gwiazda from University of California Santa Cruz will analyze uranium content in blood, kidney and lung from the same animals.

REPORTABLE OUTCOMES

A poster with data from the acute uranium exposures was presented at "Society of Toxicology Annual Meeting" in Baltimore in March 2004. The abstract is attached as Appendix 2. A poster abstract with full analysis of kidney and lung pathology from the acute uranium exposures has been submitted for the "Society of Toxicology Annual Meeting" in 2005. The abstract is attached as Appendix 3. A manuscript on these data is also in

preparation for submission to a peer-reviewed journal. The draft of this manuscript is attached as Appendix 1. Preliminary data were also presented by invitation to the Veterans' Administration Research Committee in February.

CONCLUSIONS

When interpreting the results from the ongoing Year 3 exposure in combination with exposures from Years 1-2, several patterns emerge:

First, exposure to uranium or endotoxin do not appear to affect bodyweight or brain weight of animals. Secondly, using the current method of evaluating brain uranium concentration (PIXE) we have only been able to detect uranium in a few animals. The rats showing uranium concentrations above the minimum detection level (MDL, typically 2-3 mg/kg) were either exposed for 1 day or 30 days to 1 mg/m^3 UO_2+UO_3. In these animals, the detectable uranium level was very close to the MDL. Inflammation resulting from endotoxin instillation was present in two of the three animals where CNS deposition was observed. The brain regions where uranium was above MDL were the glomeruli and mitral cells in the olfactory bulb, indicating that uranium deposition may be a consequence of direct neuronal uptake from the nasal cavity. However, the small percentage of brains with detectable uranium deposition makes it very difficult to elucidate whether uranium deposition is time- or concentration-dependent. Although the observed levels were low, we have confidence in the results. Given the number of analyses that have been performed, and the range of the MDLs in those analyses, the observed values are clearly above background. In addition, the exceedance of background has only occurred in those brain regions most likely to reflect initial uptake, i.e. the glomeruli where the olfactory neurons make their first synapse, and in one instance at the mitral cells where a subsequent synaptic connection occurs. This research was undertaken to look for a model for an illness that does not occur in 100% of exposed individuals, nor are the symptom profiles consistent across individuals. Therefore, this finding in only some animals remains important and could potentially provide an understanding of at least a component of Gulf War Illness.

The finding that the toxicity occurring in association with high-dose, acute exposures endures for up to one year in these animals is also a notable result. Damage to kidneys from uranium exposures has often been reported to reverse upon cessation of exposure. In these studies, the initial deposition in kidney produced a tubular necrosis severe enough to produce a uremic pneumonia that resulted in fibrotic changes in the lungs that persisted even if the damage to the kidney resolved. Females were substantial more sensitive to the initial kidney toxicity and related uremic pneumonia observed in these exposures. Because so many of the females died at the early time points, we made the decision to use the remaining females in the earlier sacrifices to follow any lasting changes. Therefore, we were not able to evaluate the long-term impacts of the more sensitive group as no females survived to the six-month and one-year sacrifice points. It is also of note that the toxicity occurred with the most soluble uranium compound, UO_3, but not with the UO_3 in an equal mixture with UO_2. The same degree of toxicity observed in the UO_3 exposed animals did, however, occur following exposure to depleted uranium oxide. This finding suggests that something other than solubility is contributing to the toxicity of the DU used in these studies. In the next year we hope to follow-up on this observation by a more complete analysis of the DU material.

The inconclusive results regarding brain uranium uptake also makes it difficult to determine which exposure scenarios that are most relevant for immunohistochemical analysis of the brain. Brain sectioning and histochemistry is very labor intensive and we will therefore use results from the sub-dissected, homogenized brains currently being analyzed by our

collaborator Dr. Fernando Cardozo-Pelaez in conjunction with the results of the PIXE analysis from the additional Clean-up Scenario animals and the Re-exposure animals to obtain more information regarding exposure scenarios and sacrifice timepoints most likely to lead to neuronal damage and neuroinflammation. Immunohistochemical analysis of a subset of the rats will then be performed to more systematically understand the underlying mechanisms and resulting toxicity. In the exposures completed under the rescoping, we have been able to use our original data to fine-tune the analyses, allowing us to reduce the planned PIXE analyses, thereby eliminating the need to quick-freeze brain tissues. This, in combination with the addition of animals to existing groups, allows us to paraformaldehyde perfuse and fix brain and spinal cord tissue to improve the cytoarchitecture of samples used in the upcoming final analyses and there improve the quality of our results.

To date, only early timepoints post-exposure have been evaluated for spinal cord damage. In our initial analysis we did not detect a loss of large spinal motor neurons, a feature that would be considered as severe damage (and happen at a later timepoint post-exposure). To detect more subtle neuronal damage, cervical and lumbar spinal cord will be immunohistochemically stained for inflammatory and neuronal degeneration markers. In addition, tissue obtained 6 months after inhalation of uranium aerosols will be evaluated and compared to the spinal cords harvested immediately after end of exposure. Again, paraformaldehyde perfused and fixed tissues will be used in these assessments to improve the quality of the sections used in these analysis and eliminate artifacts that hampered interpretation of pilot data.

Finally, addition of more sensitive analytic methods for kidney and lung uranium analysis should allow us to follow-up more adequately on our findings of kidney and lung toxicity. This new collaboration will also allow for analysis of blood to improve our understanding of the kinetics of redistribution and clearance following these lower-dose longer exposures.

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Appendix 1

Draft kidney and lung pathology manuscript

Pathology of Inhalation of Uranium Oxide Aerosols

Exposure for 15 minutes to 500 mg/m^3

Methods

A necropsy was performed on all rats dying, sacrificed in a moribund condition or sacrificed at scheduled times. Organs of the cranial, thoracic, and abdominal cavities were examined for grossly visible lesions. Samples of larynx, trachea, lung, bronchial lymph node, and kidney were examined for lesions by light microscopy. Formalin-fixed tissues samples were embedded in paraffin, sectioned at 5 micra, and stained with hematoxylin and eosin. Some sections were stained with Masson's trichrome to delineate fibrosis. The microscopic findings in the kidney and lung were graded using the criteria show in the Appendix (Tables A and B). A complete tabulation of the histologic findings for each rat is also shown in the Appendix (Tables C–H).

Early Deaths

Fifteen of 30 rats exposed to UO_3, the most soluble of the compounds used, died or were sacrificed moribund 2–13 days after inhalation exposure (Table 1). One male rat died 2 days after inhalation exposures with no obvious gross lesions and no histologic lesions in the kidney, lung, larynx, trachea or bronchial lymph node. The cause of death for this rat was undetermined.

Fourteen rats (12 females and 2 males) had acute tubular necrosis of the kidney involving primarily the pars recta portion of the proximal renal tubules. The tubular lining epithelium was in various stages of degeneration and necrosis. Many of the epithelial cells were necrotic and many had sloughed into the lumen (Figure 1). The tubular lumens were filled with casts of necrotic cells, cellular debris and proteinaceous casts. Some of the necrotic debris contained a granular blue staining, most likely calcium (Figure 2). A number of the necrotic tubules were lined by a few flattened epithelial cells. Occasionally the tubules with sloughed epithelium were lined with small basophilic epithelial cells of regenerating epithelium. The acute renal tubular necrosis with death within days after exposure is consistent with renal uranium-induced toxicity previously described in rats (Barnett and Metcalf, 1949; Haley, 1982).

All rats with renal tubular necrosis also had an acute, diffuse, suppurative pneumonia that was considered related to uremia. The pneumonia was moderate to marked in 12 of the 14 with tubular necrosis. The pulmonary lesion was characterized by widespread infiltration of neutrophilic inflammatory cells in the septa and alveoli, numerous focal proliferations of alveolar epithelial lining cells, focal accumulations of alveolar edema and focal fibroblastic organization of edema fluids (Figure 3). There was no concentration of inflammation around airways. In addition, calcium precipitates were seen in the alveolar septa and necrotic debris in one rat. The paucity of calcium in the tissues of most rats may be related to the rapid onset of the uremia. Hypercalcemia secondary to hyperparathyroidism is considered necessary for calcium deposition in the tissues in uremia and would not develop in a matter of days (Grassi et al., 1994).

No lesions were present in the larynx or trachea. Most of the bronchial lymph nodes had a minimal histiocytosis, probably related to the pneumonia.

Table 1. UO Study: Histologic Lesion Summary – Early Deaths and Moribund Sacrifices

Exp. No.	Animal No.	Death Type / DPE	Expose to	Larynx	Trachea	BLN Histio-cytosis	Kidney Nephro-pathy	Kidney Tubular Necrosis	Lung Uremic Pneumonia	Lung AM Hyper.
7212	C007	ND/8	UO_3	–	M	M	–	4	3	–
	C008	ND/13	UO_3	M	–	M	–	3	1	–
	C010	ND/2	UO_3	–	–	–	–	–	–	–
7213	C017	ND/10	UO_3	–	M	M	–	3	3	–
	C018	ND/6	UO_3	M	–	2	–	4	4	–
	C019	MSAC/7	UO_3	–	–	M	–	3	4	–
	C020	ND/6	UO_3	–	–	M	–	3	3	–
	C021	MSAC/7	UO_3	–	–	2	–	3	4	–
	C022	ND/6	UO_3	–	–	M	–	4	4	–
	C023	ND/6	UO_3	–	–	M	–	4	3	–
	C024	ND/4	UO_3	–	–	1	–	4	2	–
	C025	ND/6	UO_3	–	–	2	–	4	3	–
	C027	ND/8	UO_3	–	–	M	–	4	4	–
	C028	ND/7	UO_3	–	–	M	–	4	4	–
	C029	ND/8	UO_3	–	–	1	–	4	4	–

DPE = Days Post Exposure; ND = Natural Death; MSAC = Moribund Sacrifice; M = Tissue Missing
Severity: 1 = Minimal; 2 = Mild, 3 = Moderate, 4 = Marked

Zero Day Sacrifices

Fifty-six rats were sacrificed 4 hours after the end of inhalation exposure, as scheduled. Nephropathy was a minimal lesion seen in nine rats, 8 males and 1 female (Table 2). The lesion as characterized by a few proximal tubules tubules with flattened, basophilic epithelium. Hyaline casts were occasionally present. Nephropathy, a spontaneous renal disease of rats, has been well described (Montgomery and Seely, 1990). It is characterized initially a glomerular hyalinization that leads to a focal tubular regeneration and thickened basement membranes in the proximal convoluted tubules (Figure 4). The thickened the basement is accentuated by the Masson trichrome staining applied to the tissues of some cases. Mononuclear cells may accompany the change. With time more tubules and glomeruli are involved leading to marked glomerular hyalinization and sclerosis, tubular hyaline casts, interstitial fibrosis, mineralization and cyst formation. The initial lesions can occur as early as 3 months of age and may progress to renal failure as early as 12–15 months of age. The incidence and severity of the interstitial lesions are greater in males than females (Short and Goldstein, 1992).

Only minimal histologic changes were evident in the lungs of most of the exposed rats (Table 2). Minimal increases in numbers of alveolar macrophages were found in nearly all of the rats exposed to particles. A few small dark round particles could be seen in the cytoplasm of alveolar macrophages of some of the rats exposed to the more insoluble compounds, UO_2, TaO_2, and DUOx. The intranasal instillation administration of endotoxin 48 hours before the inhalation exposure did not effect the presence of particles in macrophages. A minimal increase in the number of alveolar macrophages was noted in most of the rats exposed to compounds in a relatively insoluble particulate form (UO_2, TaO_2, and DUO_x).

A broncho-interstitial pneumonia was present in six of the 18 rats intranasally instilled with endotoxin 2 days before sacrifice (Figure 5). The pneumonia was moderate to marked in four of the six rats indicating involvement of more that 25% of the lung. Cellular infiltrates of neutrophils and mononuclear cells localized around distal airways dominated the lesion. This distribution and the

nature of the cellular infiltrates differed from that of the uremic pneumonia noted in the rats that died. The pneumonia was undoubtedly a reaction to endotoxin that passed from the nasal cavity to the lung (Harkema and Hotchkiss, 1992).

No lesions were found in the larynx, trachea or bronchial lymph nodes. However, particles were seen microscopically on the tracheal epithelium in one rat exposed to TaO_2.

Table 2. Histologic Lesion Summary – 4-Hour Post Exposure Sacrifice

			Kidney		Lung					
			Nephropathy		AM Particles		AM Hyperplasia		Broncho-interstitial Pneumonia	
Exposure	Endo-toxin	Sex	Inc.	Sev.	Inc.	Sev.	Inc.	Sev.	Inc.	Sev.
Air	N	M	0/2	–	0/2	–	0/2	–	0/2	–
	N	F	0/2	–	0/2	–	0/2	–	0/2	–
UO_2	N	M	1/3	0.33	3/3	1.0	2/3	0.66	0/3	–
	N	F	0/3	–	2/3	0.66	2/3	0.66	0/3	–
UO_3	N	M	2/3	0.66	0/3	–	3/3	1.0	0/3	–
	N	F	0/3	–	0/3	–	2/3	0.66	0/3	–
$UO_2 + UO_3$	N	M	0/3	–	0/3	–	3/3	1.0	0/3	–
	N	F	0/3	–	2/3	0.66	3/3	1.0	0/3	–
TaO_2	N	M	1/3	0.33	1/3	0.33	0/3	–	0/3	–
	N	F	0/3	–	3/3	1.0	2/3	0.66	0/3	–
DUO_x	N	M	1/3	0.33	1/3	0.33	1/3	0.33	0/3	–
	N	F	1/3	0.33	2/3	0.66	2/3	0.66	0/3	–
Air	N	M	0/2	–	0/2	–	0/2	–	0/2	–
	N	F	0/2	–	0/2	–	1/2	0.5	0/2	–
$UO_2 + UO_3$	Y	M	1/3	0.33	2/3	0.66	1/3	0.33	0/3	–
	Y	F	1/3	0.33	1/3	0.33	3/3	1.33	3/3	2.66
Air	Y	M	2/3	0.66	0/3	–	3/3	1.0	1/3	0.66
	Y	F	0/3	–	0/3	–	2/3	0.66	2/3	2.33
DUO_x	Y	M	0/3	–	0/3	–	3/3	1.0	0/3	–
	Y	F	0/3	–	3/3	1.0	3/3	1.0	0/3	–

Inc. = # with lesion/# in group; Sev. = total severity score/# in group

30-Day Sacrifices

Fifty-four rats were sacrificed 30 days after the end of inhalation exposure, as scheduled. Minimal nephropathy was present in 14 rats (Table 3). It was present in 2 of the 7 air-exposed males and none of the 7 air-exposed females. A lesion classified as mild nephropathy was noted in the three males rats exposed to UO_3. The male rats exposed to the uranium compounds (UO_2, UO_3, $UO_2 + UO_3$, and DUO_x) had an incidence of 61% and the females 22%. This incidence was much higher than the incidence in rats exposed to air only or TaO_2: males 20% and females 0%.

A striking histologic lesion in the lung was a focal septal fibrosis that occurred in the scattered septa in the mid portion of the left lung (the only lung lobe available for examination) (Figure 6). The fibrosis consisted of focal accumulations of fibrous tissue that appear to be on the surfaces of the septa and alveolar ducts (Figure 7). A few mononuclear inflammatory cells accompanied the fibrosis, but were a minor feature. The septal fibrosis occurred only in those rats exposed to the uranium compounds (Table 3). Eighty to 100% of the rats exposed to UO_3 and DUO_x had minimal to mild septal fibrosis. None of the rats exposed to UO_2 and only 2 of 12 exposed to $UO_2 + UO_3$ had fibrosis. Males and females and those exposed to endotoxin were similarly affected.

Alveolar macrophages were increased in numbers in nearly all the rats exposed to particles. The incidence was greater than at the 0 day sacrifice. These macrophages contained particles and in greater numbers than at the 0 day sacrifices. The exception was the rats exposed to UO_3 in which not particles were seen in the alveolar macrophages.

No lesions were found in the larynx, trachea or bronchial lymph nodes.

Table 3. Histologic Lesion Summary – 30-Days Post Exposure Sacrifice

			Kidney		Lung					
			Nephropathy		AM Particles		AM Hyperplasia		Septal Fibrosis	
Exposure	Endo-toxin	Sex	Inc.	Sev.	Inc.	Sev.	Inc.	Sev.	Inc.	Sev.
Air	N	M	1/2	0.50	0/2	–	0/2	–	0/2	–
	N	F	0/2	–	0/2	–	1/2	0.50	0/2	–
UO_2	N	M	2/3	0.66	3/3	1.0	2/3	0.66	0/3	–
	N	F	0/3	–	3/3	1.0	2/3	0.66	0/3	–
UO_3	N	M	3/3	2.0	0/3	–	3/3	1.2	3/3	1.2
	N	F	1/1	1.0	0/1	–	1/1	2.0	1/1	2.0
$UO_2 + UO_3$	N	M	3/3	1.0	1/3	0.33	3/3	2.0	0/3	–
	N	F	0/3	–	1/3	0.33	3/3	1.7	0/3	–
TaO_2	N	M	0/3	–	3/3	1.0	2/3	0.66	0/3	–
	N	F	0/3	–	3/3	1.0	1/3	0.33	0/3	–
DUO_x	N	M	0/3	–	3/3	1.3	3/3	1.3	3/3	1.3
	N	F	1/3	0.33	2/2	1.0	2/2	2.0	2/2	1.5
Air	N	M	1/2	0.50	0/2	–	0/2	–	0/2	–
	N	F	0/2	–	1/2	0.50	0/2	–	0/2	–
$UO_2 + UO_3$	Y	M	1/3	0.33	1/3	0.33	3/3	1.3	1/3	0.66
	Y	F	1/3	0.33	1/3	0.33	3/3	1.3	1/3	0.66
Air	Y	M	0/3	–	0/3	–	0/3	–	0/3	–
	Y	F	0/3	–	0/3	–	0/3	–	0/3	–
DUO_x	Y	M	2/3	0.66	2/3	0.66	3/3	1.3	2/3	1.0
	Y	F	1/3	0.33	3/3	1.0	3/3	1.0	3/3	1.3

Inc. = # with lesion/# in group; Sev. = total severity score/# in group

180-Day Sacrifices

Fifty-three rats were sacrificed 180 days after the end of inhalation exposure, as scheduled (Table 4). No female rats exposed to UO_3 were remaining for sacrifice at 180 days because to the large number of early deaths in this group. In addition two rats exposed to DUO_x died at 135 days after exposure and one rat exposed to endotoxin was sacrificed moribund at 240 day after exposure.

A minimal nephropathy was noted in 4/18 male rats and 1/15 females rats exposed to the uranium compounds and in 2/10 males and 0/10 females exposed to air or TaO_2. This incidence in the uranium compound exposed rats (22% in males and 6.6% in females) was much less than the incidence in uranium-compound exposed rats sacrificed at 30 days after inhalation exposure. In contrast, the incidence in the air or TaO_2 rats was similar to that in rats sacrificed at 30 days.

The septal fibrosis, first seen at the 30-day sacrifice, was still present in most of the rats exposed to UO_3 or DUO_x and in one female exposed to $UO_2 + UO_3$. None was present in rats exposed to UO_2. The severity of the fibrosis was slightly less and accompanying mononuclear cells were minimal compared to rats sacrificed at 30 days. Hyperplasia of alveolar macrophages and the presence of particles in the macrophages were slightly less in incidence and severity compared to rats sacrificed at 30 days after exposure.

No lesions were found in the larynx, trachea or bronchial lymph nodes.

Table 4. Histologic Lesion Summary – 180-Days Post Exposure Sacrifice

			Kidney		Lung					
			Nephropathy		AM Particles		AM Hyperplasia		Septal Fibrosis	
Exposure	Endo-toxin	Sex	Inc.	Sev.	Inc.	Sev.	Inc.	Sev.	Inc.	Sev.
Air	N	M	0/2	–	0/2	–	0/2	–	0/2	–
	N	F	0/2	–	0/2	–	1/2	0.50	0/2	–
UO_2	N	M	0/3	–	3/3	1.3	3/3	1.0	0/3	–
	N	F	0/3	–	3/3	1.3	2/3	1.0	0/3	–
UO_3	N	M	0/3	–	0/3	–	2/3	1.0	3/3	1.0
	N	F	None available for sacrifice							
$UO_2 + UO_3$	N	M	2/3	0.66	1/3	0.33	2/3	0.66	0/3	–
	N	F	0/3	–	2/3	0.66	3/3	1.0	0/3	–
TaO_2	N	M	1/3	0.33	0/3	–	0/3	–	0/3	–
	N	F	0/3	–	1/3	0.33	1/3	0.33	0/3	–
DUO_x	N	M	0/3	–	3/3	1.0	3/3	1.3	3/3	1.0
	N	F	1/3	0.33	3/3	1.0	3/3	2.3	3/3	1.3
Air	N	M	0/2	–	0/2	–	0/2	–	0/2	–
	N	F	0/2	–	0/2	–	1/2	0.50	0/2	–
$UO_2 + UO_3$	Y	M	0/3	–	0/3	–	1/3	0.33	0/3	–
	Y	F	0/3	–	1/3	0.33	2/3	1.0	1/3	0.33
Air	Y	M	1/3	0.33	0/3	–	2/3	0.66	0/3	–
	Y	F	0/3	–	0/3	–	1/3	0.33	0/3	–
DUO_x	Y	M	2/3	0.66	0/3	–	1/3	0.33	1/3	0.33
	Y	F	0/3	–	1/3	0.33	3/3	1.3	3/3	1.3

Inc. = # with lesion/# in group; Sev. = total severity score/# in group

360-Day Sacrifices

Eighty-seven rats were sacrificed 360 days after the end of inhalation exposure, as scheduled (Table 5). No female rats exposed to UO_3 were remaining for sacrifice at 360 days because to the large number of early deaths in this group. In addition, one rat exposed to TaO_2 died 340 days after exposure.

A minimal to mild nephropathy was noted in 25/37 male rats and 2/30 females rats exposed to the uranium compounds and in 10/20 males and 1/20 females exposed to air or TaO_2. This incidence in the uranium compound exposed rats (68% in males and 6.6% in females) was similar to the incidence in the air or TaO_2 rats (50% in males and 5% in females) sacrificed at 360 days.

The septal fibrosis, seen at the 30- and 180-day sacrifice, was still present in most of the rats exposed to UO_3 or DUO_x and in one female exposed to $UO_2 + UO_3$. In addition, one male exposed to UO_2 had septal fibrosis. The severity of the fibrosis was slightly less and accompanying mononuclear cells were minimal compared to rats sacrificed at 30 days. Hyperplasia of alveolar macrophages and the presence of particles in the macrophages were reduced in incidence and severity compared to rats sacrificed at 180 days after exposure. In addition, a number of rats had minimal alveolar histiocytosis, characterized by small, focal accumulations of alveolar macrophages, typically sub-adjacent to the pleura. The air-exposed and UO_2-exposed rats had a higher incidence than rats exposed to the other compounds. Alveolar histiocytosis is a not uncommon lesion in aging rats (Montgomery and Seely, 1990b).

No lesions were found in the larynx, trachea or bronchial lymph nodes.

Table 5. Histologic Lesion Summary – 360-Days Post Exposure Sacrifice

			Kidney		Lung							
			Nephropathy		AM Particles		AM Hyperplasia		Septal Fibrosis		Histio-cytosis	
Exposure	Endo-toxin	Sex	Inc.	Sev.	Inc.	Sev.	Inc.	Sev.	Inc.	Sev.	Inc.	Sev.
Air	N	M	2/4	0.75	0/4	–	0/4	–	0/4	–	3/4	1.0
	N	F	0/4	–	0/4	–	0/4	–	0/4	–	0/4	–
UO_2	N	M	4/7	0.57	5/7	0.71	0/7	–	1/7	0.14	0/7	–
	N	F	0/6	–	4/6	0.66	0/6	–	0/6	–	2/6	-.33
UO_3	N	M	3/4	0.75	0/4	–	0/4	–	4/4	1.5	0/4	–
	N	F	None available for sacrifice									
$UO_2 + UO_3$	N	M	6/7	0.86	0/7	–	0/7	–	0/7	–	0/7	–
	N	F	0/7	–	0/7	–	0/7	–	1/7	0.14	1/7	0.14
TaO_2	N	M	3/6	0.50	0/6	–	1/6	0.17	0/6	–	2/6	0.33
	N	F	1/7	0.29	0/7	–	0/7	–	0/7	–	2/7	0.29
DUO_x	N	M	5/7	0.71	1/7	0.29	4/7	0.57	6/7	0.86	0/7	–
	N	F	1/7	0.29	1/7	0.29	5/7	0.71	6/7	1.0	1/7	0.29
Air	N	M	0/4	–	0/4	–	0/4	–	0/4	–	2/4	0.50
	N	F	0/4	–	0/4	–	0/4	–	0/4	–	1/4	0.25
$UO_2 + UO_3$	Y	M	4/6	0.66	0/6	–	0/6	–	0/6	–	0/6	–
	Y	F	0/6	–	0/6	–	0/6	–	0/6	–	0/6	–
Air	Y	M	5/6	0.83	0/6	–	0/6	–	0/6	–	0/6	–
	Y	F	0/5	–	0/5	–	0/5	–	0/5	–	0/5	–
DUO_x	Y	M	3/6	0.50	2/6	0.33	2/6	0.33	6/6	1.0	0/6	–
	Y	F	1/4	0.25	2/4	0.50	0/4	–	4/4	1.0	0/6	–

Inc. = # with lesion/# in group; Sev. = total severity score/# in group

Late Deaths

Three rats died or were sacrificed moribund135 to 240 days after inhalation exposure (Appendix Table G). Two females, exposed to endotoxin and DUOx were found dead 135 days after exposure. Minimal septal fibrosis was present in one of the rats. Neither rat had nephropathy. A female exposed to endotoxin was found moribund 240 days after exposure and one male exposure to TaO_2 was found dead 340 days after exposure. No lung or kidney lesions were found in these two rats. Causes of death were not determined in any of the four rats.

Discussion

Acute renal tubular necrosis resulting in death within 14 days was found after inhalation of high concentrations of the relatively soluble uranium compound UO_3. Females were much more susceptible than males. Uremic pneumonia was significant lesion that was present in all the rats that died of renal toxicity. The pneumonia was severe enough to cause or contribute to the deaths. The females, with smaller lungs, may not have been able to handle the inflammation as well as the larger males, resulting in a higher incidence of deaths. Deaths due to renal toxicity did not occur with the other uranium compounds.

At 30 days after inhalation exposure, renal tubular necrosis was not present in any of the rats. However, the incidence of a lesion classified as nephropathy was greatly increased in both males and females exposed to the uranium compounds when compared with the air or TaO_2 exposed rats where no renal toxicity would be expected (Table 6). Much of the lesion in the uranium-exposed rats was probably related to repair of the tubules following the acute renal tubular necrosis which could not be differentiated histologically from the spontaneous lesion of nephropathy. The incidence of nephropathy at 180 and 360 days after exposure is essentially the same as the air and TaO_2-exposed rats, which can be considered the background for spontaneous nephropathy. This indicates that a non-fatal acute tubular necrosis probably occurred in about 40% of the males and 20% of the females exposed to the uranium compounds, including DUO_x.

Table 6. Incidence of Nephropathy

	Days After Inhalation Exposure							
	4 Hours		30 Days		180 Days		360 Days	
	M	F	M	F	M	F	M	F
UO_2	1/3 33%	0/3	2/3 66%	0/3	0/3	0/3	4/7 57%	0/6
UO_3	2/3 66%	0/3	3/3 100%	1/1 100%	0/3	na	3/4 75%	na
$UO_2 + UO_3$	1/6 17%	1/6 17%	4/6 66%	1/6 17%	2/6 33%	0/6	10/13 77%	0/13
DUO_x	1/6 17%	1/6 17%	2/6 33%	2/6 33%	2/6 33%	1/6 17%	8/13 62%	2/11 18%
All U	5/18 28%	2/18 11%	11/18 61%	4/18 22%	4/18 22%	1/15 6.6%	25/37 37%	2/30 6.6%
TaO_2	1/3 33%	0/3 0	0/3 0	0/3 0	1/3 33%	0/3 0	3/6 50%	1/7 14%
Air	2/7 29%	0/7 0	2/7 29%	0/7 0	1/7 14%	0/7 0	7/14 50%	0/13 0
All Non U	3/10 30%	0/10 0	2/10 20%	0/10 0	2/10 20%	0/10 0	10/20 50%	1/20 5%

The pulmonary septal fibrosis seen 30 days after exposure in the rats exposed to several of the uranium compounds is a novel finding (Table 7). Two unusual features of the fibrosis were the focal nature of the lesion and the distribution in the central portion, not the cranial or caudal portions of the left lung. The lesion was seen in 100% of the rats surviving the UO_3 exposure and in 67% to 100% of the rats exposed to DUO_x. It was not seen in any of the air or TaO_2-exposed rats.

The septal fibrosis is considered to be sequelae of uremic pneumonia. This is based on the indication that rats exposed to UO_3, the group that had the highest incidence and severity of fibrosis, was also the group that had rats dying with uremic pneumonia. It is plausible to think that the surviving rats also had a uremic pneumonia. In addition, the distribution of the fibrosis in the lung is unusual. There are no available reports on the distribution or sequelae of uremic pneumonia in rats. However, the distribution of fibrosis is not similar to that of inhaled particles (Dungworth et al., 1995). Such fibrosis is centered on the distal airways, is more uniform in distribution within the lung and is usually involves the interstitium to some degree.

Table 7. Incidence of Pulmonary Fibrosis

	Days after Inhalation Exposure					
	30 Days		180 Days		360 Days	
	M	F	M	F	M	F
UO_2	0/3	0/3	0/3	0/3	1/7 14%	0/6
UO_3	3/3 100%	1/1 100%	3/3 100%	na	4/4 100%	na
$UO_2 + UO_3$	1/6 17%	1/6 17%	0/6	1/6 17%	0/13	1/13 7.7%
DUO_x	5/6 83%	5/5 100%	4/6 67%	6/6 100%	12/13 92%	10/11 91%
All U	9/18 50%	7/15 47%	7/18 39%	7/15 47%	17/27 63%	11/30 35%
TaO_2	0/3	0/3	0/3	0/3	0/6	0/7
Air	0/7	0/7	0/7	0/7	0/14	0/13
All Non U	0/10	0/10	0/10	0/10	0/20	0/20

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Appendix

Table A. UO Studies: Histologic Criteria for Grading Severity of Kidney Lesions

Diagnosis	Grade	Criteria*
Tubular necrosis	–	No evidence of tubular necrosis
	Minimal	Few, scattered foci of tubular necrosis; slough of tubular epithelium in pars recta of proximal tubule (P3 segment)
	Mild	Mild tubular necrosis in pars recta, sloughing of cells, hyaline casts; involves 5–25% of the proximal tubules
	Moderate	Moderate, tubular necrosis in pars recta, sloughing of cells, granular and cellular casts; involves 25–50% of the proximal tubules
	Marked	Marked, tubular necrosis in the pars recta, sloughing of cells, hyaline casts, mineralization; involves >50% of the proximal tubules
Nephropathy	–	No evidence of nephropathy
	Minimal	Few, scattered foci of contiguous tubules with regeneration, thickened basement membranes and karyomegaly; may be hyaline casts in tubules; located in the proximal convoluted tubules (P1, P2 segments)
	Mild	Mild tubular regeneration, hyaline casts and cellular infiltrates; involves <25% of the cortical parenchyma
	Moderate	Moderate, tubular, and glomerular fibrosis; involves 25–50% of the cortical parenchyma; hyaline casts in medulla
	Marked	Marked, tubular, and glomerular fibrosis; involves >50 % of the cortical parenchyma; hyaline casts in medulla

*Local severity may increase grade one level over grade determined by distribution.

Table B. UO Studies: Histologic Criteria for Grading Severity of Lung Lesions

Diagnosis	Grade	Criteria*
Particle loading of alveolar macrophages (AMs)	–	Essentially no particles in scant cytoplasm
	Minimal	A few black particles scattered in cytoplasm or a few macrophages with abundant particles
	Mild	Moderate number of particles in cytoplasm (≤10); do not obscure nucleus of macrophage
	Moderate	Many particles (too many to count) in cytoplasm cover the nucleus; slightly enlarged cytoplasm
Alveolar macrophage hyperplasia	–	Few scattered AMs in alveoli; difficult to find
	Minimal	Minimal increase in number of AMs
	Mild	Mild increase in number of AMs; easily found at high magnification; average 1/alveolus
	Moderate	Moderate increase in number of AMs; easily found at low magnification; several macrophages/alveolus
Broncho-interstitial pneumonia	–	No inflammation present
	Minimal	A few inflammatory cells infiltrating septa around bronchioles; involves <10% of lung
	Mild	Infiltrating inflammatory cells involving 10–25% of the lung; may also be in alveoli
	Moderate	Infiltrating inflammatory cells involving 26–50% of the lung; also in alveoli
	Marked	Infiltrating inflammatory cells involving >50% of lung; also in alveoli
Uremic pneumonia	–	No inflammation present
	Minimal	Edema and a few (PMNs) in alveoli; involves <10% of lung
	Mild	Edema and PMNs in alveoli; calcium (Ca) present; involves 10–25% of lung
	Moderate	Edema and PMNs in alveoli; Ca present; involves 26–50% of lung
	Marked	Edema and PMNs in alveoli; Ca present; involves >50% of lung
Fibrosis, septal	–	No fibrosis present
	Minimal	Slight to moderate thickening of alveolar septa with collagen; involves <10% of lung
	Mild	Thickening of alveolar septa with collagen; involves 10–25% of lung
	Moderate	Thickening of alveolar septa easily recognizable; involves 26–50% of lung

*Local severity may increase grade one level over grade determined by distribution.

Table C. UO Study: Histologic Lesion Summary – Early Deaths and Moribund Sacrifices

Exp. No.	Animal No.	Death Type / DPE	Expose to	Larynx	Trachea	BLN	Kidney		Lung	
						Histio-cytosis	Nephro-pathy	Tubular Necrosis	Uremic Pneumonia	AM Hyper.
7212	C007	ND/8	UO_3	–	M	M	–	4	3	–
	C008	ND/13	UO_3	M	–	M	–	3	1	–
	C010	ND/2	UO_3	–	–	–	–	–	–	–
7213	C017	ND/10	UO_3	–	M	M	–	3	3	–
	C018	ND/6	UO_3	M	–	2	–	4	4	–
	C019	MSAC/7	UO_3	–	–	M	–	3	4	–
	C020	ND/6	UO_3	–	–	M	–	3	3	–
	C021	MSAC/7	UO_3	–	–	2	–	3	4	–
	C022	ND/6	UO_3	–	–	M	–	4	4	–
	C023	ND/6	UO_3	–	–	M	–	4	3	–
	C024	ND/4	UO_3	–	–	1	–	4	2	–
	C025	ND/6	UO_3	–	–	2	–	4	3	–
	C027	ND/8	UO_3	–	–	M	–	4	4	–
	C028	ND/7	UO_3	–	–	M	–	4	4	–
	C029	ND/8	UO_3	–	–	1	–	4	4	–

DPE = Days Post Exposure; ND = Natural Death; MSAC = Moribund Sacrifice; M = Tissue Missing
Severity: 1 = Minimal; 2 = Mild, 3 = Moderate, 4 = Marked

Table D. UO Study: Histologic Summary – 4-Hour Sacrifice

						Kidney	Lung		
Exp. No.	Animal No.	Exposure Compound	Larynx	Trachea	BLN	Nephro-pathy	AM Particles	AM Hyper.	Broncho-interstitial Pneumonia
7208	A009	None	–	–	–	–	–	–	–
	A010	None	–	–	M	–	–	–	–
7209	A019	None	–	–	–	–	–	–	–
	A020	None	–	–	M	–	–	–	–
7210	B014	UO_2	–	–	–	1	2	2	–
	B015	UO_2	–	–	–	1	1	1	–
	B016	UO_2	–	–	M	1	1	–	–
7211	B030	UO_2	–	–	–	–	–	1	–
	B031	UO_2	–	–	–	–	1	–	–
	B032	UO_2	–	–	–	–	1	1	–
7212	C014	UO_3	–	–	M	1	–	1	–
	C015	UO_3	–	–	–	1	–	1	–
	C016	UO_3	–	–	–	1	–	1	–
7213	C030	UO_3	M	–	M	–	–	–	–
	C031	UO_3	–	–	–	–	–	1	–
	C032	UO_3	–	–	–	–	–	1	–
7214	D014	$UO_2 + UO_3$	M	–	–	–	–	1	–
	D015	$UO_2 + UO_3$	–	–	–	1	–	1	–
	D016	$UO_2 + UO_3$	–	–	M	–	–	1	–
7215	D030	$UO_2 + UO_3$	–	–	–	–	1	2	–
	D031	$UO_2 + UO_3$	–	–	–	–	1	1	–
	D032	$UO_2 + UO_3$	–	–	M	–	–	1	–
7216	E014	TaO_2	–	particles	M	–	–	–	–
	E015	TaO_2	–	–	M	1	–	–	–
	E016	TaO_2	–	–	M	–	1	–	–
7217	E030	TaO_2	–	M	M	–	1	–	–
	E031	TaO_2	M	–	M	–	1	1	–
	E032	TaO_2	M	–	M	–	1	1	–
7218	F014	DUO_x	–	–	–	–	1	1	–
	F015	DUO_x	–	–	–	1	–	–	–
	F016	DUO_x	–	–	–	–	–	–	–
7219	F030	DUO_x	–	–	M	–	–	–	–
	F031	DUO_x	–	–	M	–	1	1	–
	F032	DUO_x	–	–	–	1	1	1	–

Table D. UO Study: Histologic Summary – 4-Hour Sacrifice (Concluded)

						Kidney	Lung		
Exp. No.	Animal No.	Exposure Compound	Larynx	Trachea	BLN	Nephro-pathy	AM Particles	AM Hyper.	Broncho-interstitial Pneumonia
7220	G009	Air	–	–	–	–	–	–	–
	G010	Air	–	–	–	–	–	–	–
7221	G019	Air	–	–	–	–	–	–	–
	G020	Air	–	–	M	–	–	1	–
7222	I013	$UO_2 + UO_3$/ Endo	–	–	–	–	1	–	–
	I014	$UO_2 + UO_3$/ Endo	–	–	M	–	–	1	–
	I015	$UO_2 + UO_3$/ Endo	–	–	–	1	1	–	–
7223	I028	$UO_2 + UO_3$/ Endo	–	–	–	–	1	2	3
	I029	$UO_2 + UO_3$/ Endo	–	–	–	–	–	–	2
	I030	$UO_2 + UO_3$/ Endo	–	–	–	–	–	–	3
7224	J013	Endo	–	–	–	–	–	1	–
	J014	Endo	–	–	–	1	–	1	2
	J015	Endo	–	–	–	1	–	1	–
7225	J028	Endo	–	–	–	–	–	1	4
	J029	Endo	–	–	–	–	–	–	–
	J030	Endo	–	–	–	–	–	1	3
7226	K013	DUO_x/Endo	–	–	–	–	–	1	–
	K014	DUO_x/Endo	–	–	–	1	–	1	–
	K015	DUO_x/Endo	–	–	–	–	–	1	–
7227	K028	DUO_x/Endo	–	–	–	–	1	1	–
	K029	DUO_x/Endo	–	–	M	–	1	1	–
	K030	DUO_x/Endo	–	–	M	–	1	1	–

Severity: 1 = Minimal; 2 = Mild, 3 = Moderate, 4 = Marked; M = Tissue Missing

Table E. UO Study: Histologic Summary – 30-Day Sacrifice

			Larynx	Trachea	BLN	Kidney	Lung		
Exp. No.	Animal No.	Exposure Compound	Chronic Inflam.	Chronic Inflam.	Histio-cytosis	Nephro-pathy	AM Particles	AM Hyper.	Fibrosis - Septal
7208	A007	None	–	–	–	1	–	–	–
	A008	None	–	–	M	–	–	–	–
7209	A017	None	–	–	–	–	–	–	–
	A018	None	–	–	M	–	–	1	–
7210	B011	UO_2	1	–	–	–	1	–	–
	B012	UO_2	–	–	–	1	1	1	–
	B013	UO_2	–	–	–	1	1	2	–
7211	B027	UO_2	–	–	–	–	1	1	–
	B028	UO_2	–	–	1	–	1	–	–
	B029	UO_2	–	–	–	–	1	1	–
7212	C011	UO_3	–	–	–	2	–	3	2
	C012	UO_3	–	–	M	2	–	3	3
	C013	UO_3	1	1	–	2	–	1	2
7213	C026	UO_3	1	–	–	1	–	2	2
7214	D011	$UO_2 + UO_3$	–	–	M	1	–	2	–
	D012	$UO_2 + UO_3$	–	–	–	1	1	2	–
	D013	$UO_2 + UO_3$	–	–	–	1	–	2	–
7215	D027	$UO_2 + UO_3$	2	–	–	–	–	2	–
	D028	$UO_2 + UO_3$	–	–	1	–	–	2	–
	D029	$UO_2 + UO_3$	–	–	–	–	1	1	–
7216	E011	TaO_2	–	–	M	–	1	1	–
	E012	TaO_2	M	–	–	–	1	1	–
	E013	TaO_2	–	–	M	–	1	–	–
7217	E027	TaO_2	–	–	–	–	1	1	–
	E028	TaO_2	1	–	–	–	1	–	–
	E029	TaO_2	–	–	–	–	1	–	–
7218	F011	DUO_x	1	–	–	–	1	1	1
	F012	DUO_x	–	–	–	–	2	2	2
	F013	DUO_x	1	–	–	–	1	1	1
7219	F027	DUO_x	–	M	M	–	M	M	M
	F028	DUO_x	–	–	–	–	1	2	2
	F029	DUO_x	–	–	M	1	1	2	1
7220	G007	Air/Endo	–	–	–	–	–	–	–
	G008	Air/Endo	–	–	–	1	–	–	–

Table E. UO Study: Histologic Summary – 30-Day Sacrifice (Concluded)

			Larynx	Trachea	BLN	Kidney	Lung		
Exp. No.	Animal No.	Exposure Compound	Chronic Inflam.	Chronic Inflam.	Histio-cytosis	Nephro-pathy	AM Particles	AM Hyper.	Fibrosis - Septal
7221	G017	Air/Endo	M	M	M	–	1	–	–
	G018	Air/Endo	–	–	–	–	–	–	–
7222	I010	$UO_2 + UO_3$/ Endo	–	–	–	–	–	1	–
	I011	$UO_2 + UO_3$/ Endo	1	–	–	–	1	2	2
	I012	$UO_2 + UO_3$/ Endo	–	–	–	1	–	1	–
7223	I025	$UO_2 + UO_3$/ Endo	M	M	M	–	–	1	–
	I026	$UO_2 + UO_3$/ Endo	–	–	–	–	1	1	–
	I027	$UO_2 + UO_3$/ Endo	1	–	–	–	–	1	–
7224	J010	Endo	1	–	–	–	–	–	–
	J011	Endo	1	–	–	–	–	–	–
	J012	Endo	–	–	–	–	–	–	–
7225	J025	Endo	–	–	–	–	–	–	–
	J026	Endo	–	–	–	–	–	–	–
	J027	Endo	–	–	–	–	–	–	–
7226	K010	DUO_x/Endo	1	–	M	–	–	1	1
	K011	DUO_x/Endo	1	–	–	1	1	2	2
	K012	DUO_x/Endo	–	–	–	1	1	1	–
7227	K025	DUO_x/Endo	–	–	–	–	1	1	2
	K026	DUO_x/Endo	M	–	–	–	1	1	1
	K027	DUO_x/Endo	–	–	–	1	1	1	1

Severity: 1 = Minimal; 2 = Mild, 3 = Moderate, 4 = Marked; M = Tissue Missing

Table F. UO Study: Histologic Summary – 180-Day Sacrifice

			Larynx	Trachea	BLN	Kidney	Lung		
Exp. No.	Animal No.	Exposure Compound	Chronic Inflam.	Chronic Inflam.	Histio-cytosis	Nephro-pathy	AM Particles	AM Hyper.	Fibrosis - Septal
7208	A005	None	–	–	–	–	–	–	–
	A006	None	–	–	1	–	–	–	–
7209	A015	None	M	M	M	–	–	–	–
	A016	None	–	–	–	–	–	1	–
7210	B008	UO_2	–	–	–	–	2	1	–
	B009	UO_2	1	–	–	–	1	1	–
	B010	UO_2	–	–	–	–	1	1	–
7211	B023	UO_2	–	–	1	–	2	2	–
	B024	UO_2	1	–	M	–	1	1	–
	B025	UO_2	–	–	1	–	1	–	–
7212	C005	UO_3	2	–	M	–	–	2	1
	C006	UO_3	–	–	–	–	–	–	1
	C009	UO_3	–	–	M	–	–	1	1
7214	D018	$UO_2 + UO_3$	–	–	1	1	1	1	–
	D019	$UO_2 + UO_3$	–	1	1	–	–	1	–
	D020	$UO_2 + UO_3$	–	1	–	1	–	–	–
7215	D024	$UO_2 + UO_3$	–	–	–	–	–	1	–
	D025	$UO_2 + UO_3$	–	–	1	–	1	1	–
	D026	$UO_2 + UO_3$	–	–	–	–	1	1	–
7216	E018	TaO_2	1	–	–	1	–	–	–
	E019	TaO_2	–	–	M	–	–	–	–
	E020	TaO_2	–	–	–	–	–	–	–
7217	E024	TaO_2	–	–	–	–	–	–	–
	E025	TaO_2	1	–	1	–	–	–	–
	E026	TaO_2	–	–	1	–	1	1	–
7218	F008	DUO_x	–	–	1	–	1	1	1
	F009	DUO_x	–	–	–	–	1	1	1
	F010	DUO_x	–	–	–	–	1	2	1
7219	F024	DUO_x	–	–	–	–	1	2	1
	F025	DUO_x	–	–	1	–	1	2	1
	F026	DUO_x	–	–	1	1	1	3	2
7220	G005	Air/Endo	–	–	–	–	–	–	–
	G006	Air/Endo	2	–	–	–	–	–	–
7221	G014	Air/Endo	–	–	M	–	–	1	–
	G015	Air/Endo	–	–	–	–	–	–	–

Table F. UO Study: Histologic Summary – 180-Day Sacrifice (Concluded)

			Larynx	Trachea	BLN	Kidney	Lung		
Exp. No.	Animal No.	Exposure Compound	Chronic Inflam.	Chronic Inflam.	Histio-cytosis	Nephro-pathy	AM Particles	AM Hyper.	Fibrosis - Septal
7222	I007	$UO_2 + UO_3$/ Endo	–	–	–	–	–	–	–
	I008	$UO_2 + UO_3$/ Endo	–	–	–	–	–	–	–
	I009	$UO_2 + UO_3$/ Endo	–	–	–	–	–	1	–
7223	I022	$UO_2 + UO_3$/ Endo	–	–	–	–	–	–	–
	I023	$UO_2 + UO_3$/ Endo	–	–	1	–	–	1	–
	I024	$UO_2 + UO_3$/ Endo	–	–	M	–	1	2	1
7224	J007	Endo	–	–	–	–	–	1	–
	J008	Endo	–	–	–	1	–	–	–
	J009	Endo	–	–	–	–	–	1	–
7225	J022	Endo	1	–	1	–	–	–	–
	J023	Endo	–	–	–	–	–	1	–
	J024	Endo	–	–	M	–	–	–	–
7226	K007	DUO_x/Endo	2	–	1	–	–	–	–
	K008	DUO_x/Endo	1	–	1	1	–	–	1
	K009	DUO_x/Endo	1	–	1	1	–	1	–
7227	K022	DUO_x/Endo	I	–	M	–	–	1	1
	K023	DUO_x/Endo	1	–	–	–	1	2	2
	K024	DUO_x/Endo	–	–	–	–	–	1	1

Severity: 1 = Minimal; 2 = Mild, 3 = Moderate, 4 = Marked; M = Tissue Missing

Table G. UO Study: Histologic Summary – 360-Day Sacrifice

			BLN	Kidney	Lung			
Exp. No.	Animal No.	Exposure Compound	Histio-cytosis	Nephro-pathy	AM Particles	AM Hyper.	Fibrosis - Septal	Histio-cytosis
7208	A001	None	–	–	–	–	–	1
	A002	None	–	–	–	–	–	1
	A003	None	–	2	–	–	–	–
	A004	None	–	1	–	–	–	2
7209	A011	Air	–	–	–	–	–	–
	A012	Air	–	–	–	–	–	–
	A013	Air	–	–	–	–	–	–
	A014	Air	–	–	–	–	–	–
7210	B001	UO_2	–	–	1	–	–	–
	B002	UO_2	–	2	–	–	–	–
	B003	UO_2	–	1	–	–	1	–
	B004	UO_2	–	–	1	–	–	–
	B005	UO_2	–	–	1	–	–	–
	B006	UO_2	1	1	1	–	–	–
	B007	UO_2	–	1	1	–	–	–
7211	B017	UO_2	–	–	–	–	–	–
	B018	UO_2	–	–	–	–	–	1
	B019	UO_2	3	–	1	–	–	–
	B020	UO_2	–	–	1	–	–	–
	B021	UO_2	–	–	1	–	–	–
	B022	UO_2	–	–	1	–	–	1
7212	C001	UO_3	–	1	–	–	2	–
	C002	UO_2	–	2	–	–	1	–
	C003	UO_2	–	–	–	–	1**	–
	C004	UO_2	–	1	–	–	2	–
7214	D001	$UO_2 + UO_3$	1	-	–	–	–	–
	D002	$UO_2 + UO_3$	2	1	–	–	–	–
	D003	$UO_2 + UO_3$	–	1	–	–	–	–
	D004	$UO_2 + UO_3$	–	1	–	–	–	–
	D005	$UO_2 + UO_3$	–	1	–	–	–	–
	D006	$UO_2 + UO_3$	1	1	–	–	–	–
	D007	$UO_2 + UO_3$	–	2	–	–	–	–

Table G. UO Study: Histologic Summary – 360-Day Sacrifice (Continued)

			BLN	Kidney	Lung			
Exp. No.	Animal No.	Exposure Compound	Histio-cytosis	Nephro-pathy	AM Particles	AM Hyper.	Fibrosis - Septal	Histio-cytosis
7215	D017	$UO_2 + UO_3$	–	–	–	–	1**	–
	D018	$UO_2 + UO_3$	–	–	–	–	–	–
	D019	$UO_2 + UO_3$	–	–	–	–	–	–
	D020	$UO_2 + UO_3$	–	–	–	–	–	–
	D021	$UO_2 + UO_3$	1	–	–	–	–	1
	D022	$UO_2 + UO_3$	–	–	–	–	–	–
	D023	$UO_2 + UO_3$	–	–	–	–	–	–
7216	E001	TaO_2	–	1	–	2	–	–
	E002	TaO_2	2	1	–	–	–	1
	E003	TaO_2	I	–	–	–	–	–
	E004	TaO_2	I	1	–	–	–	–
	E005	TaO_2	1	–	–	–	–	–
	E006	TaO_2	–	–	–	–	–	1
7217	E017	TaO_2	–	–	–	–	–	–
	E018	TaO_2	1	–	–	–	–	–
	E019	TaO_2	1	–	–	–	–	1
	E020	TaO_2	–	–	–	–	–	–
	E021	TaO_2	–	–	–	–	–	–
	E022	TaO_2	–	–	–	–	–	1
	E023	TaO_2	–	2	–	–	–	–
7218	F001	DUO_x	–	–	–	1	1	–
	F002	DUO_x	–	1	–	1	1	–
	F003	DUO_x	–	1	–	–	–	–
	F004	DUO_x	–	1	–	–	1	–
	F005	DUO_x	1	1	–	1	1	–
	F006	DUO_x	M	–	–	–	1	–
	F007	DUO_x	1	1	1	1	1	–
7219	F017	DUO_x	–	–	1	1	1	–
	F018	DUO_x	–	–	–	1	2	–
	F019	DUO_x	1	1	–	1	1	–
	F020	DUO_x	–	–	–	1	1	1
	F021	DUO_x	–	–	–	–	1	–
	F022	DUO_x	–	–	–	1	1	–
	F023	DUO_x	–	–	–	–	–	–

Table G. UO Study: Histologic Summary – 360-Day Sacrifice (Continued)

			BLN	Kidney	Lung			
Exp. No.	Animal No.	Exposure Compound	Histio-cytosis	Nephro-pathy	AM Particles	AM Hyper.	Fibrosis - Septal	Histio-cytosis
7220	G001	Air/Endo	1	–	–	–	–	–
	G002	Air/Endo	1	–	–	–	–	1
	G003	Air/Endo	M	–	–	–	–	1***
	G004	Air/Endo	–	–	–	–	–	–
7221	G011	Air/Endo	1	–	–	–	–	–
	G012	Air/Endo	–	–	–	–	–	1**
	G013	Air/Endo	–	–	–	–	–	–
	G016	Air/Endo	M	–	–	–	–	–
7222	I001	$UO_2 + UO_3$/ Endo	1	1	–	–	–	–
	I002	$UO_2 + UO_3$/ Endo	–	1	–	–	–	–
	I003	$UO_2 + UO_3$/ Endo	–	–	–	–	–	–
	I004	$UO_2 + UO_3$/ Endo	–	–	–	–	–	–
	I005	$UO_2 + UO_3$/ Endo	1	1	–	–	–	–
	I006	$UO_2 + UO_3$/ Endo	–	2	–	–	–	–
7223	I016	$UO_2 + UO_3$/ Endo	1	–	–	–	–	–
	I017	$UO_2 + UO_3$/ Endo	–	–	–	–	–	–
	I018	$UO_2 + UO_3$/ Endo	–	–	–	–	–	–
	I019	$UO_2 + UO_3$/ Endo	1	–	–	–	–	–
	I020	$UO_2 + UO_3$/ Endo	2	–	–	–	–	–
	I021	$UO_2 + UO_3$/ Endo	–	–	–	–	–	–

Table G. UO Study: Histologic Summary – 360-Day Sacrifice (Concluded)

			BLN	Kidney	Lung			
Exp. No.	Animal No.	Exposure Compound	Histio-cytosis	Nephro-pathy	AM Particles	AM Hyper.	Fibrosis - Septal	Histio-cytosis
7224	J001	Endo	1	1	–	–	–	–
	J002	Endo	–	1	–	–	–	–
	J003	Endo	–	1	–	–	–	–
	J004	Endo	–	1	–	–	–	–
	J005	Endo	1	1	–	–	–	–
	J006	Endo	–	–	–	–	–	–
7225	J016	Endo	–	–	–	–	–	–
	J018	Endo	–	–	–	–	–	–
	J019	Endo	1	–	–	–	–	–
	J020	Endo	1	–	–	–	–	–
	J021	Endo	1	–	–	–	–	–
7226	K001	DUO_x/Endo	1	–	–	1	1	–
	K002	DUO_x/Endo	1	–	–	–	1	–
	K003	DUO_x/Endo	1	1	–	–	1	–
	K004	DUO_x/Endo	M	1	1	–	1	–
	K005	DUO_x/Endo	M	1	–	–	1	–
	K006	DUO_x/Endo	–	–	1	1	1	–
7227	K018	DUO_x/Endo	1	–	–	–	1	–
	K019	DUO_x/Endo	1	–	1	–	1	–
	K020	DUO_x/Endo	1	1	1	–	1	–
	K021	DUO_x/Endo	–	–	–	–	1	–

Severity: 1 = Minimal; 2 = Mild, 3 = Moderate, 4 = Marked; M = Tissue Missing
*Also pleural fibrosis (2), **Also focal epithelial hyperplasia (3), ***Also granuloma (1)

Table H. UO Study: Histologic Lesion Summary – Late Deaths

Exp. No.	Animal No.	Death Type / DPE	Expose to	Larynx	Trachea	BLN	Kidney	Lung		
				Chronic Inflam.	Chronic Inflam.	Histio-cytosis	Nephro-pathy	AM Particles	AM Hyper.	Fibrosis - Septal
7216	E007	ND/340	TaO_2	1	–	1	–	–	–	–
7225	J017	MSAC/240	Endo	–	–	1	–	–	–	–
7227	K016	ND/135	DUO_x/ Endo	–	–	M	–	1	1	–
	K017	ND/135		–	–	–	–	1	–	1

DPE = Days Post Exposure; ND = Natural Death; MSAC = Moribund Sacrifice; M = Tissue Missing
Severity: 1 = Minimal; 2 = Mild, 3 = Moderate, 4 = Marked

Pathology of Inhalation of Uranium Oxide Aerosols:

Figures

Figure 1
Acute renal tubular necrosis with necrotic debris in tubular lumen in a rat sacrificed moribund 7 days after inhalation exposure to UO_3. (C021, 20x, H&E stain)

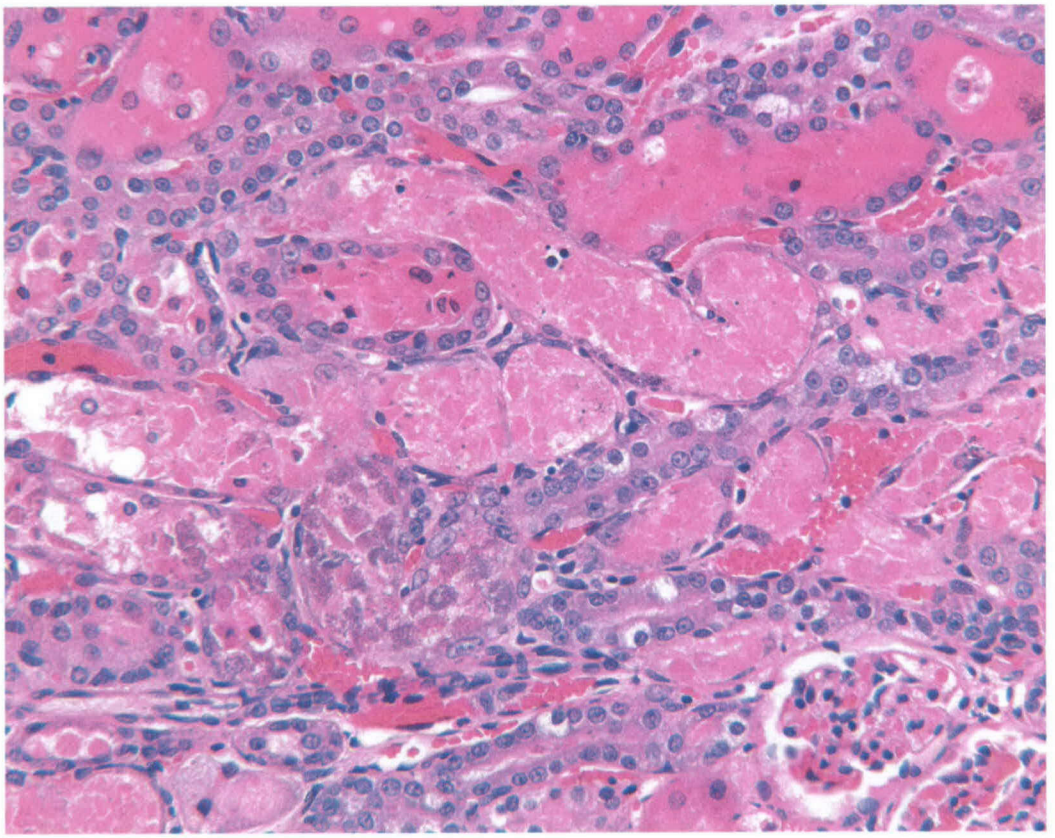


Figure 2
Necrotic, calcified debris and hyaline casts in tubules of rat dying 10 days after inhalation exposure to UO_3. Flattened epithelial cells line one tubule. (C017, 20x, H&E stain)

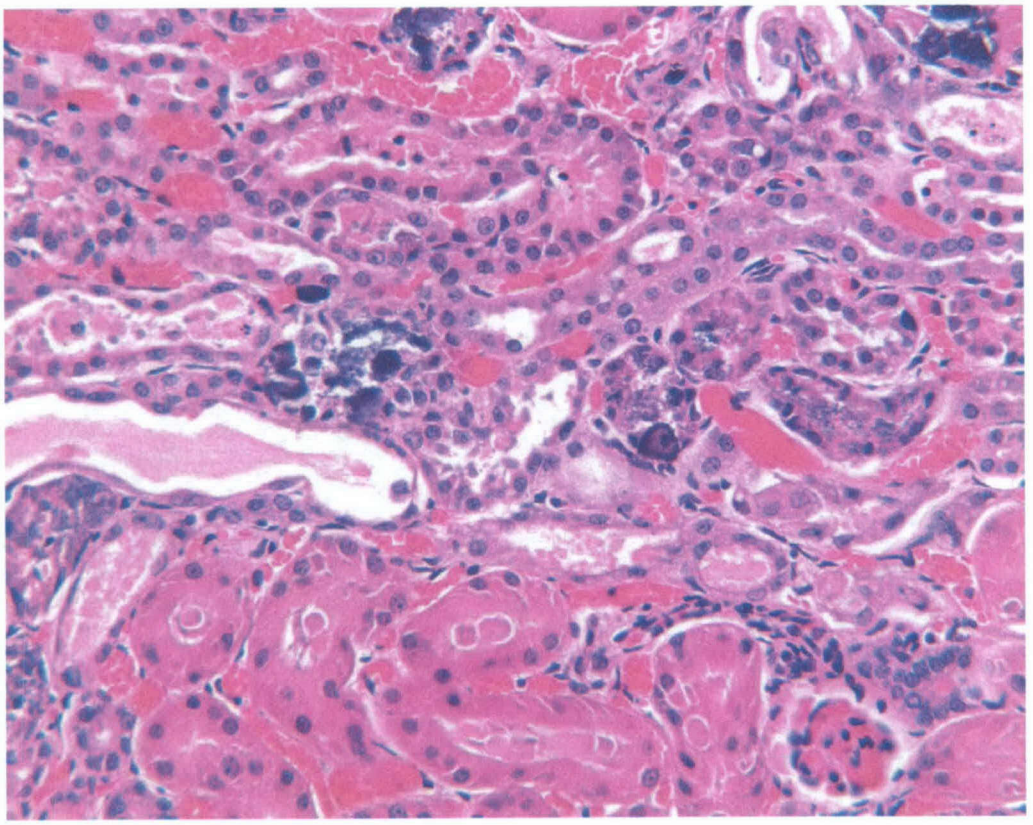


Figure 3
Uremia pneumonia in a rat sacrificed moribund 7 days after inhalation exposure to UO_3. Inflammatory cells in the alveolar interstitium, exudate in alveolar lumen, early fibroblastic organization of alveolar exudate and focal proliferation of alveolar epithelial cells. (C021, 20x, H&E stain)

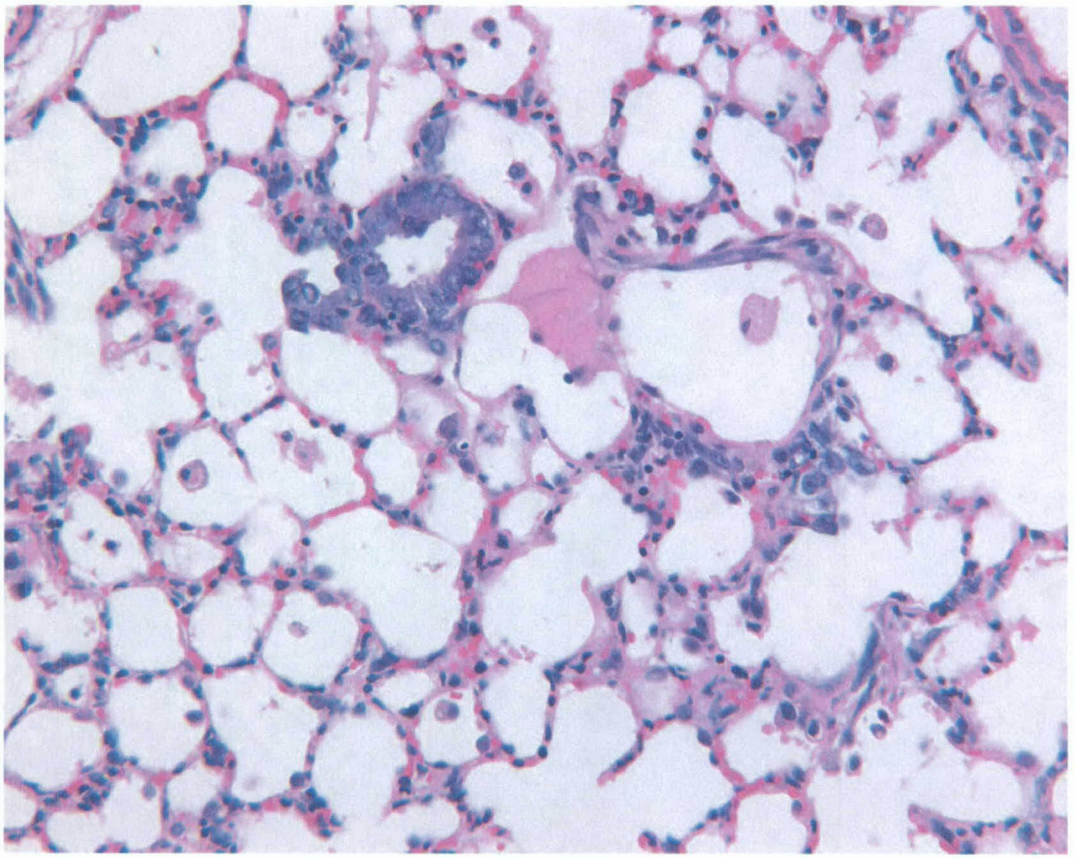


Figure 4
Nephropathy in a rat sacrificed 2 hours after inhalation exposure to TaO_2. A group of proximal tubules with regenerating epithelium (E015, 20x, H&E stain)

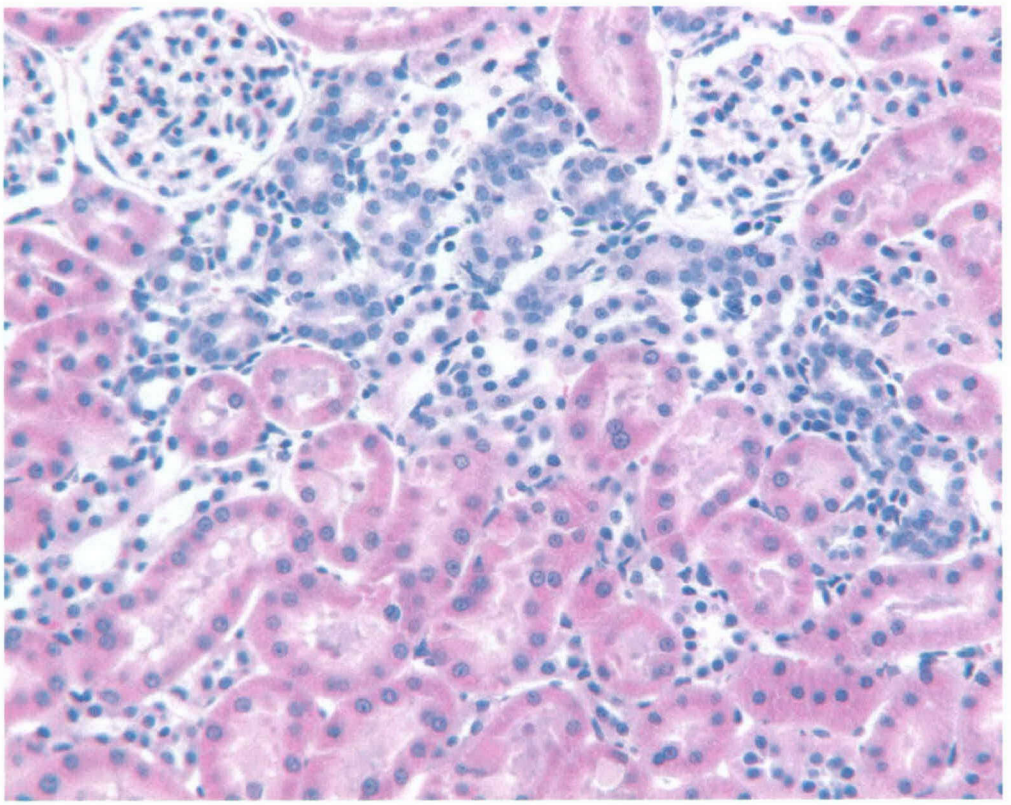


Figure 5

Broncho-interstitial pneumonia in a rat sacrificed 2 days after endotoxin instillation and 4 hrs after inhalation exposure to air only. Inflammatory cells in the interstitium around the bronchiole and extending into the alveoli. (I028, 10x, H&E stain)

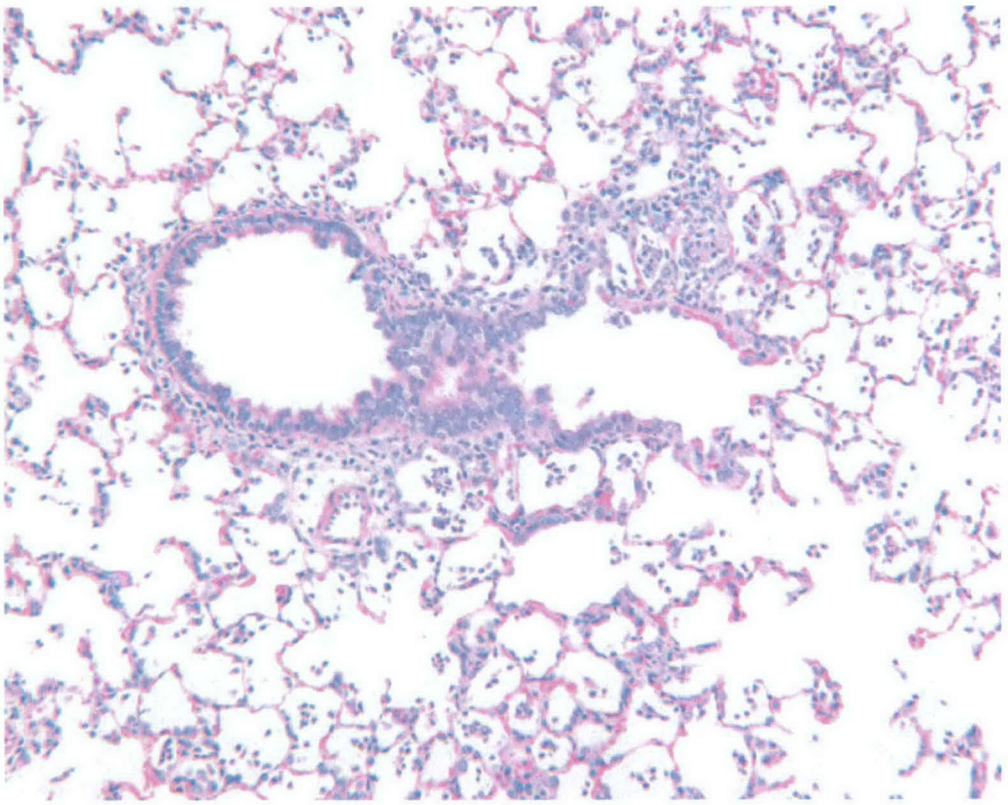


Figure 6

Distribution of septal fibrosis in a rat sacrificed 30 days after inhalation exposure to UO_3. Septal thickenings with fibrosis are noted on the right side of the figure. (C013, 2x, H&E stain)

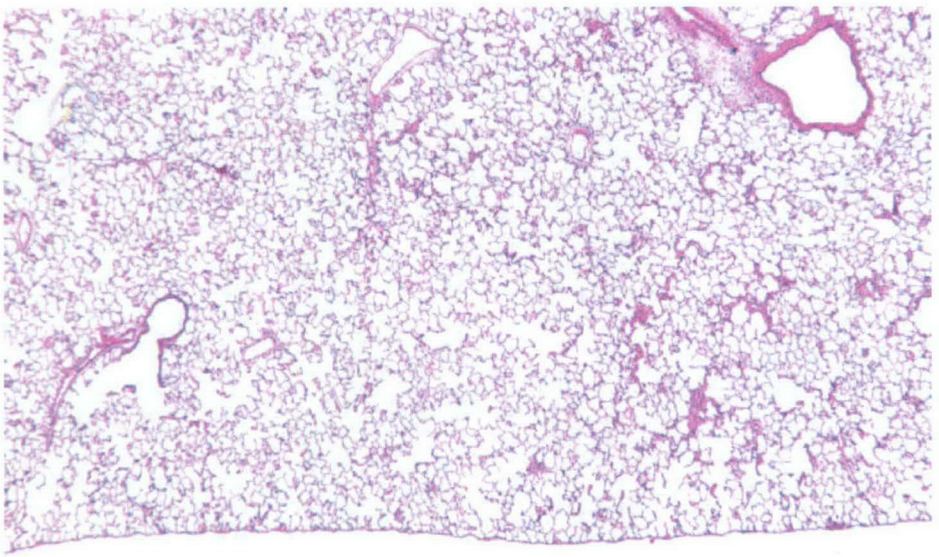
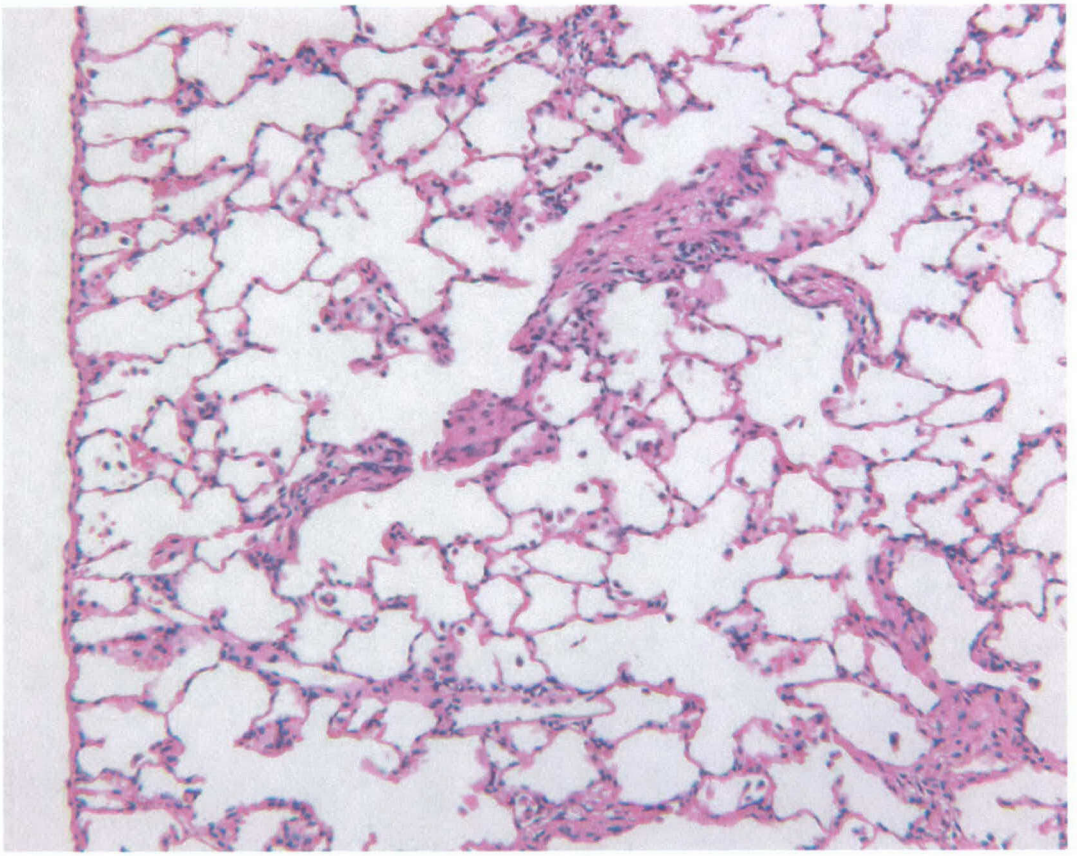


Figure 7
Focal alveolar septal fibrosis in a rat sacrificed 30 days after inhalation exposure to UO_3. Large accumulations of fibrous tissue line the alveolar duct and septa (C012, 10x, H&E stain)



Appendix 2

INHALATION OF URANIUM OXIDE: PHYSIOLOGICAL EFFECTS ON RATS

Lewis, Johnnye[1]; Karlsson, Jenny[1]; Bench, Graham[3]; Myers, Orrin[1]; Barrington, Wendy[1]; Barr, Edward[2]; Hahn, Fletcher[2]

1. Community Environmental Health Program, University of New Mexico, Albuquerque, NM, USA.
2. Lovelace Respiratory Research Institute, Albuquerque, NM, USA.
3. Lawrence Livermore National Laboratory, Livermore, CA, USA.

Depleted uranium (DU) has been implicated as a potential factor contributing to Gulf War illness. Upon impact, DU-containing armor penetrators burn and release uranium dust particles of respirable size. Inhaled metals can be directly transported into the brain via the olfactory system, and inflammation in nasal epithelium may increase brain metal uptake further. We therefore investigated the deposition of uranium in brain and kidney as well as neuroinflammatory response in olfactory bulb. Rats were exposed to either: a) insoluble UO2, b) soluble UO3, c) 50% UO2 + 50% UO3, d) DU oxide (DUOx), e) TaO2 (negative control) or f) air (control) for 15 min in a nose-only inhalation chamber. The metal concentration in aerosols ranged from 300-600 mg/m3 and particle size was 1.5-2.4 μm. Nasal inflammation was induced in a subset of animals by endotoxin instillation in both nostrils 48 hours prior to air, DUOx or UO2 + UO3 exposure as above. Animals were sacrificed on day of exposure (0 day) or 30 days post-exposure for tissue analysis of uranium content. Astroglial response in olfactory bulbs at he same timepoints was evaluated using glial fibrillary acidic protein (GFAP) immunoreactivity. Of the 30 rats exposed to UO3, 12 females and 3 male rats died within 12 days after exposure and were found to have acute renal tubular necrosis and uremic pneumonia. There were no early deaths in other experimental groups. Uranium levels in kidneys as well as brain olfactory system were below the detectable level at 0 and 30 days in all experimental groups. Nevertheless, GFAP intensity in olfactory bulb glomeruli was significantly elevated in UO3 exposed animals at 0 and 30 days compared to air-exposed control rats. There was a trend for DUOx to increase GFAP response at 0 days. Thus, uranium oxide can, even at levels too low for detection in brain, lead to neuroinflammation. Effect of uranium on other tissue types and brain regions is currently under investigation.

Appendix 3

Biologic Effects After Brief Inhalation Exposure to Uranium Oxide or Depleted Uranium Oxide Aerosols

Fletcher F. Hahn[1], Edward B. Barr[1], Graham Bench[2], Jenny Karlsson[3] and Johnnye Lewis[3] Lovelace Respiratory Research Institute[1], Albuquerque, NM, Lawrence Livermore National Laboratory[2], Livermore CA and University of New Mexico[3], Albuquerque, NM

To study of the transport of inhaled uranium oxides to the brain, rats were exposed to aerosols of UO_2, UO_3, $UO_2 + UO_3$ or depleted UO_x (DUOx). Control groups were exposed to TaO2 or air only. The uranium compounds were purchased as 99.8% purity and the DUOx was prepared by heating DU 0.75% titanium metal to a 30 mesh powder and then ball-milling. Groups of 30 rats each were exposed for 15 min. to nominal aerosol concentrations of 500mg/m^3 and scheduled for sacrifice at 2 hr., 30 180 and 365 days after end of exposure. The achieved aerosol concentrations were UO_2, 572 mg/m^3; UO_3, 329 mg/m^3; $UO_2 + UO_3$, 302 mg/m^3 and DUOx, 609 mg/m^3. The aerosol particle sizes were all respirable, ranging from 1.6 to 2.4 μm MMAD.

Fifteen rats exposed to UO_3, the most soluble of the compounds used, died 2–13 days post inhalation exposure (d.P.E.). All but one died of acute renal tubular necrosis and uremic pneumonia, characteristic of acute uranium-induced toxicity. Rats sacrificed 2 hr. P.E. had minimal nephropathy and minimal histologic changes in the lungs, regardless of exposure. In rats sacrificed 30 d.P.E., focal septal pulmonary fibrosis was present in 80-100% of those exposed to UO_3 or DUOx. None of those exposed to UO_3 and 17% of exposed to $UO_2 + UO_3$ had pulmonary fibrosis. The incidence of nephropathy was increased in all rats exposed to UO compounds relative to the 2 hr. sacrifice. In rats sacrificed 180 or 360 d.P.E. the pattern of increased incidence of nephropathy and pulmonary fibrosis continued in those exposed to the UO compounds.

These results show that more soluble UO_3 can induce acute renal effects in rats after a brief inhalation exposure. In addition, pulmonary effects can be seen after brief inhalation of any of the UO compounds. These effects are most likely secondary to the acute renal toxicity.

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